QP
501
J7
Vol. 213

CONTENTS OF VOLUME 213

No. 1, March, 1955

PAGE

Rabinovitz, M., Olson, Margaret E., and Greenberg, David M. Relation of energy processes to the incorporation of amino acids into proteins of the Ehrlich ascites carcinoma 1

Rivoire, Raymond, Rivoire, Jane, and Poujol, Jean. The determination of urinary 17-trioxysteroids 11

Henneman, Philip H., Rourke, G. Margaret, and Jackson, W. P. U. Depression of serum alkaline phosphatase activity by human serum albumin 19

Black, Simon, and Wright, Nancy G. β-Aspartokinase and β-aspartyl phosphate 27

Black, Simon, and Wright, Nancy G. Aspartic β-semialdehyde dehydrogenase and aspartic β-semialdehyde 39

Black, Simon, and Wright, Nancy G. Homoserine dehydrogenase 51

Melville, Donald B., Horner, William H., Otken, Charles C., and Ludwig, Martha L. Studies on the origin of ergothioneine in animals 61

Sperry, Warren M., and Brand, Florence C. The determination of total lipides in blood serum 69

Asnis, Robert E. A glutathione reductase from *Escherichia coli* 77

Baggett, Billy, Engel, Lewis L., and Fielding, Linda L. The estimation of hydroxysteroids 87

Engel, Lewis L., Carter, Priscilla, and Fielding, Linda L. Urinary metabolites of administered corticosterone. I. Steroids liberated by glucuronidase hydrolysis 99

Webb, Junius M., and Levy, Hilton B. A sensitive method for the determination of deoxyribonucleic acid in tissues and microorganisms 107

Taurog, Alvin, Potter, G. D., and Chaikoff, I. L. Conversion of inorganic I^{131} to organic I^{131} by cell-free preparations of thyroid tissue 119

Renold, Albert E., Hastings, A. Baird, Nesbett, Frances B., and Ashmore, James. Studies on carbohydrate metabolism in rat liver slices. IV. Biochemical sequence of events after insulin administration 135

Mazur, Abraham, Baez, Silvio, and Shorr, Ephraim. The mechanism of iron release from ferritin as related to its biological properties 147

Segal, Harold L. Kinetic studies on the phenol-sulfate conjugating system of rat liver 161

Li, Choh Hao, Geschwind, Irving I., Dixon, Jonathan S., Levy, Anthony L., and Harris, J. Ieuan. Corticotropins (ACTH). I. Isolation of α-corticotropin from sheep pituitary glands 171

Levy, Anthony L., Geschwind, Irving I., and Li, Choh Hao. Corticotropins (ACTH). II. Amino acid composition of α-corticotropin 187

Cole, R. David, and Li, Choh Hao. Studies on pituitary lactogenic hormone. XIV. A simplified procedure of isolation 197

Olivard, Joanne, and Snell, Esmond E. Growth and enzymatic activities of vitamin B_6 analogues. I. D-Alanine synthesis 203

Olivard, Joanne, and Snell, Esmond E. Growth and enzymatic activities of vitamin B_6 analogues. II. Synthesis of miscellaneous amino acids 215

Longenecker, J. B., and Snell, Esmond E. A possible mechanism for kynureninase action 229

XUM

LINKER, ALFRED, MEYER, KARL, and WEISSMANN, BERNARD. Enzymatic formation of monosaccharides from hyaluronate 237
HAMILTON, PAUL B., and ANDERSON, ROBERTA A. Hydroxylysine: isolation from gelatin and resolution of its diastereoisomers by ion exchange chromatography 249
GREEN, MAURICE, and STAHMANN, MARK A. Preparation and enzymatic hydrolysis of poly-γ-ethylglutamyl bovine plasma albumin 259
JEDEIKIN, LILLIAN A., and WEINHOUSE, SIDNEY. Metabolism of neoplastic tissue. VI. Assay of oxidized and reduced diphosphopyridine nucleotide in normal and neoplastic tissues 271
ELWYN, DAVID, WEISSBACH, ARTHUR, HENRY, SARAH S., and SPRINSON, DAVID B. The biosynthesis of choline from serine and related compounds 281
WOOD, HENRY N., and BALLS, A. K. Enzymatic oxidation of α-chymotrypsin ... 297
HALL, LEO M., and MACVICAR, ROBERT. Ammonia as an intermediate in nitrate reduction by *Bacillus subtilis* 305
FELS, I. GORDON, SIMPSON, MIRIAM E., and EVANS, HERBERT M. Purification of the anterior hypophyseal thyrotropic hormone 311
STOKES, WILLIAM M., FISH, WILLIAM A., and HICKEY, FREDERICK C., O. P. The fate of injected cholestenone in the intact rat 325
MEINHART, JOSEPHINE O., and SIMMONDS, SOFIA. Serine metabolism in a mutant strain of *Escherichia coli* strain K-12 329
HUFFMAN, MAX N., and LOTT, MARY HARRIET. 16-Substituted steroids. XI. Estradiol-3,16β 343
WEISS, SAMUEL B., and MARX, WALTER. The fate of radioactive cholesterol in mice with modified thyroid activities 349
ABELSON, PHILIP H., and VOGEL, HENRY J. Amino acid biosynthesis in *Torulopsis utilis* and *Neurospora crassa* 355
WANG, CHIH H., CHRISTENSEN, BERT E., and CHELDELIN, VERNON H. Conversion of pyruvic acid-2-C^{14} to some aliphatic monoamino acids in yeast 365
MORRISON, MARTIN, and STOTZ, ELMER. Partition chromatography of hemins. Separation of the prosthetic groups of cytochromes *a* and a_3 373
CANELLAKIS, E. S., and COHEN, PHILIP P. On the nature of oxonic acid and allantoxaidin as oxidation products of uric acid and allantoin 379
CANELLAKIS, E. S., and COHEN, PHILIP P. The end-products and intermediates of uric acid oxidation by uricase 385
CANELLAKIS, E. S., TUTTLE, ALICE L., and COHEN, PHILIP P. A comparative study of the end-products of uric acid oxidation by peroxidases 397
SHEPHERD, JAMES A., LI, YAO WEN, MASON, EDWARD E., and ZIFFREN, SIDNEY E. The distribution of aconitase and fumarase in homogenates of human liver 405
ARMSTRONG, MARVIN D. The phenylalanine and tyrosine requirements of the rat 409
HOFMANN, KLAUS, and TAUSIG, FRED. The chemical nature of the fatty acids of a group C *Streptococcus* species 415
HOFMANN, KLAUS, and TAUSIG, FRED. On the identity of phytomonic and lactobacillic acids. A reinvestigation of the fatty acid spectrum of *Agrobacterium* (*Phytomonas*) *tumefaciens* 425
LANGNER, RALPH R., and VOLKMANN, CAROL M. The fate of ingested acetyl-L- and D-tryptophan in the human subject 433
DE MEIO, R. H., WIZERKANIUK, MARTHA, and SCHREIBMAN, I. Enzymatic system synthesizing sulfuric acid esters of phenols 439

STRITTMATTER, PHILIPP, and BALL, ERIC G. Formaldehyde dehydrogenase, a glutathione-dependent enzyme system 445
ZUCKER, MILTON, and NASON, ALVIN. A pyridine nucleotide-hydroxylamine reductase from *Neurospora* 463
RAFELSON, MAX E., JR. Conversion of radioactive glucose and acetate to tryptophan by *Aerobacter aerogenes* 479

No. 2, APRIL, 1955

LEVY, ANTHONY L., and LI, CHOH HAO. Corticotropins (ACTH). III. Kinetic studies on the reaction of *N*-terminal group reagents with α-corticotropin, and *N*-terminal group analysis 487
HARRIS, J. IEUAN, and LI, CHOH HAO. Corticotropins (ACTH). IV. The action of carboxypeptidase on α-corticotropin, and the *C*-terminal amino acid sequence 499
ROLL, PAUL M., and WELIKY, IRVING. The utilization of nucleotides by the mammal. II. N^{15}-labeled purine nucleotides 509
WEINFELD, HERBERT, ROLL, PAUL M., and BROWN, GEORGE BOSWORTH. The utilization of nucleotides by the mammal. III. Isomeric purine nucleotides labeled with C^{14} 523
TCHEN, T. T., and VENNESLAND, BIRGIT. Enzymatic carbon dioxide fixation into oxalacetate in wheat germ 533
TCHEN, T. T., LOEWUS, FRANK A., and VENNESLAND, BIRGIT. The mechanism of enzymatic carbon dioxide fixation into oxalacetate 547
MAGASANIK, BORIS. The metabolic control of histidine assimilation and dissimilation in *Aerobacter aerogenes* 557
MAGASANIK, BORIS, and BOWSER, HELEN R. The degradation of histidine by *Aerobacter aerogenes* 571
BURSTEIN, SHLOMO, and DORFMAN, RALPH I. Steroid metabolism in guinea pigs. I. Metabolism of cortisol *in vivo* 581
BURSTEIN, SHLOMO, DORFMAN, RALPH I., and NADEL, ELI M. Corticosteroids in the urine of normal and scorbutic guinea pigs: isolation and quantitative determination 597
WU, FENG CHI, and LASKOWSKI, M. Action of the naturally occurring trypsin inhibitors against chymotrypsins α and B 609
VERLY, WALTER G., and CATHEY, WILLIAM J. The influence of vitamin B_{12} on the biosynthesis of the methyl group of choline from methanol 621
ANDERSON, E. P., YEN, C. Y., MANDEL, H. GEORGE, and SMITH, PAUL K. Ureidosuccinic acid as a precursor of nucleic acid pyrimidines in normal and tumor-bearing mice 625
STROMINGER, JACK L., and LOWRY, OLIVER H. The quantitative histochemistry of brain. IV. Lactic, malic, and glutamic dehydrogenases 635
SIEGELMAN, H. W. Quercetin glycosides of Grimes Golden apple skin 647
SHULMAN, N. RAPHAEL. A proteolytic inhibitor with anticoagulant activity separated from human urine and plasma 655
SINEX, F. MAROTT, PLAZIN, JOHN, CLAREUS, D., BERNSTEIN, W., VAN SLYKE, DONALD D., and CHASE, R. Determination of total carbon and its radioactivity. II. Reduction of required voltage and other modifications 673
ARTOM, CAMILLO. Effect of choline administration on the oxidation of fatty acids by extrahepatic tissues 681
LOWENSTEIN, J. M., and COHEN, PHILIP P. Studies on the mechanism of carbamylaspartic acid synthesis 689

XUM

ZAKRZEWSKI, SIGMUND F., and NICHOL, CHARLES A. The incorporation of formate-C^{14} into *citrovorum* factor 697
SEAMAN, GERALD R., and NASCHKE, MARY DELL. Removal of thioctic acid from enzymes 705
MORTENSON, L. E., and WILSON, P. W. Metabolism of ribose-5-phosphate by *Azotobacter vinelandii* 713
STETTEN, MARJORIE R., and STETTEN, DEWITT, JR. Glycogen regeneration *in vivo* 723
PORTMAN, OSCAR W., and MANN, GEORGE V. The disposition of taurine-S^{35} and taurocholate-S^{35} in the rat: dietary influences 733
KOVACHEVICH, R., and WOOD, W. A. Carbohydrate metabolism by *Pseudomonas fluorescens*. III. Purification and properties of a 6-phosphogluconate dehydrase 745
KOVACHEVICH, R., and WOOD, W. A. Carbohydrate metabolism by *Pseudomonas fluorescens*. IV. Purification and properties of 2-keto-3-deoxy-6-phosphogluconate aldolase 757
DIMANT, E., LANDSBERG, EDITH, and LONDON, IRVING M. The metabolic behavior of reduced glutathione in human and avian erythrocytes 769
RODKEY, F. LEE. Oxidation-reduction potentials of the diphosphopyridine nucleotide system 777
YANIV, HAIM, and GILVARG, CHARLES. Aromatic biosynthesis. XIV. 5-Dehydroshikimic reductase 787
ARMSTRONG, MARVIN D., SHAW, KENNETH N. F., and ROBINSON, KATHRYN S. Studies on phenylketonuria. II. The excretion of *o*-hydroxyphenylacetic acid in phenylketonuria 797
ARMSTRONG, MARVIN D., and SHAW, KENNETH N. F. Studies on phenylketonuria. III. The metabolism of *o*-tyrosine 805
SNOKE, JOHN E. Isolation and properties of yeast glutathione synthetase 813
SNOKE, JOHN E., and BLOCH, KONRAD. Studies on the mechanism of action of glutathione synthetase 825
GLEASON, GEOFFREY I. Some notes on the exchange of iodine with thyroxine homologues 837
EDELMAN, J., GINSBURG, V., and HASSID, W. Z. Conversion of monosaccharides to sucrose and cellulose in wheat seedlings 843
BROWN, GENE M., IKAWA, MIYOSHI, and SNELL, ESMOND E. Synthesis and microbiological activity of some pantothenic acid conjugates 855
WARD, G. B., BROWN, GENE M., and SNELL, ESMOND E. Phosphorylation of pantothenic acid and pantethine by an enzyme from *Proteus morganii* 869
IKAWA, MIYOSHI, and O'BARR, JEAN S. The isolation of α,α'-diaminopimelic acid from *Lactobacillus arabinosus* 877
TROLL, WALTER, and SHERRY, SOL. The activation of human plasminogen by streptokinase 881
CERECEDO, LEOPOLD R., and EICH, STEPHEN. Studies on thiamine analogues. IV. Neopyrithiamine and oxyneopyrithiamine. A comparison of their effects *in vivo* and *in vitro* 893
RUPE, C. O., and FARMER, CHESTER J. Amino acid studies in the transformation of proteins of the hen's egg to tissue proteins during incubation 899
WICK, ARNE N., DRURY, DOUGLAS R., NAKADA, HENRY I., BARNET, HARRY N., and MORITA, TOSHIKO N. Glucosamine and the action of insulin 907

ROSE, WILLIAM C., LEACH, BYRON E., COON, MINOR J., and LAMBERT, G. FREDERICK. The amino acid requirements of man. IX. The phenylalanine requirement 913

HERBERT, EDWARD, POTTER, VAN R., and TAKAGI, YASUYUKI. Nucleotide metabolism. IV. The phosphorylation of 5′-uridine nucleotides by cell fractions from rat liver 923

HUMPHREYS, T. E., and STUMPF, P. K. Fat metabolism in higher plants. IV. Preparation of soluble fatty acid oxidases from peanut microsomes 941

HUENNEKENS, F. M., BASFORD, R. E., and GABRIO, BEVERLY WESCOTT. An oxidase for reduced diphosphopyridine nucleotide 951

WOTIZ, HERBERT H., RICHTERICH-VAN BAERLE, ROLAND, and LEMON, HENRY M. Studies in steroid metabolism. III. Metabolism of testosterone by human serum 969

INDEX TO VOLUME 213 977

RELATION OF ENERGY PROCESSES TO THE INCORPORATION OF AMINO ACIDS INTO PROTEINS OF THE EHRLICH ASCITES CARCINOMA*

By M. RABINOVITZ, MARGARET E. OLSON, and DAVID M. GREENBERG

(From the Department of Physiological Chemistry, University of California School of Medicine, Berkeley, California)

(Received for publication, September 10, 1954)

Considerable evidence has accumulated that the incorporation of amino acids into protein requires the participation of an external source of energy (1). The requirement for this energy source has been linked to that made available by oxidation. Anaerobic conditions have been reported to produce complete or variable inhibition of incorporation in several tissues (1). The possible participation of energy contributed by anaerobic glycolysis has not been fully investigated. In this communication it is shown that glycolysis can supply energy for the incorporation of several amino acids into the protein of the Ehrlich ascites carcinoma.

The nature of this energy requirement has been the subject of considerable speculation, and various postulations involving phosphorylated amino acids (2), transpeptidation reactions (3), and activated template mechanisms (4) have been suggested. An attempt is made here to define more clearly the nature of the activation process. To this end the extent of incorporation into protein of amino acids, both singly and in groups, was determined under conditions of a suboptimal energy supply brought about by the addition of 2,4-dinitrophenol.

Methods

The procedures employed were those described previously (5), with variations in the contents of the Warburg flasks and time of incubation as indicated for individual experiments. The radioactive amino acids were also the same with the addition of DL-methionine-2-C^{14} (2.1 μc. per mg.), which was obtained from Tracerlab, Inc., Boston. Non-radioactive DL-methionine was obtained from the Nutritional Biochemicals Corporation, Cleveland. The 2,4-dinitrophenol (DNP) was an Eastman Kodak product which was recrystallized twice from water. When oxygen uptake was measured, air was used in the gas phase, and 10 per cent sodium hydroxide with a filter paper strip was placed in the center well. No difference could

* Aided by grants from the Damon Runyon Memorial Fund for Cancer Research, Inc., and the Cancer Research Funds of the University of California.

XUM

be found between oxygen uptake rates with air or 100 per cent oxygen in the gas phase.

In glycolysis experiments the buffer used for the incubation was a modified Krebs-Ringer-phosphate-bicarbonate medium of the following composition: 0.10 M sodium chloride, 1.3×10^{-2} M potassium chloride, 1.8×10^{-3} M calcium chloride, 6.5×10^{-4} M magnesium chloride, 0.02 M sodium bicarbonate, and sodium phosphate buffer, 5×10^{-3} M, pH 7.4. The gas phase was 95 per cent nitrogen and 5 per cent carbon dioxide. No difference in the incorporation of leucine into protein was observed between incubations carried out in the Krebs-Ringer-phosphate buffer medium with air as the gas phase and in the phosphate-bicarbonate medium with 95 per cent oxygen and 5 per cent carbon dioxide as the gas phase.

RESULTS AND DISCUSSION

Activation of Amino Acid Incorporation by Anaerobic Glycolysis—The incorporation of amino acids *in vitro* into the proteins of a number of tissues was reported to be inhibited by anaerobiosis (1). In most of these studies neither was glucose added nor was the rate of glycolysis determined. LePage suggested that anaerobic glycolysis can support the incorporation of glycine into the protein of several tissues (6). However, half of the activity of the isolated proteins was lost after performate oxidation. This activity may have been due in large part to disulfide linkage with radioactive glutathione, for it is known that anaerobic glycolysis can furnish the energy necessary for glutathione synthesis (7). More recently Zamecnik and Keller have reported (8) that a rat liver particulate preparation could support the incorporation of leucine into protein for short periods under anaerobic conditions if glycolytic intermediates were added to the incubation medium.

As shown in Table I, anaerobic conditions without added glucose completely abolished the incorporation of leucine, valine, lysine, phenylalanine, and methionine into the proteins of the Ehrlich ascites carcinoma. When sufficient glucose was present, the glycolysis rate was linear for 30 to 40 minutes, but tapered off slightly afterward (Fig. 1). When the glucose concentration was decreased below that necessary to maintain glycolysis for 1 hour, the initial glycolysis rate remained constant until an equivalent amount of acid was produced. In addition, no difference in the incorporation of leucine was observed when the glucose concentration was increased 6-fold above that necessary for optimal glycolysis. Under conditions of optimal glucose concentration the incorporation of amino acids varied from 60 to 72 per cent of that supported by oxidation of endogenous substrate. The specific activity of the protein remained constant upon performate oxidation.

If the concentration of glucose was decreased to permit glycolysis to continue for only a portion of the incubation period, the incorporation of leucine was lower than would be anticipated from the proportional extent of glycolysis. This is evident from the nature of the induction period observed for incorporation under anaerobic conditions (Fig. 1). When the concentration of glucose was not sufficiently high to maintain glycolysis beyond that required for the induction period (about 15 minutes), the termination of incorporation coincided with that of glycolysis. The cause of the induction period for incorporation is not certain at present, but it is of interest to note that this period did not occur under the usual conditions

TABLE I

Oxidation-Linked and Anaerobic Glycolysis-Linked Incorporation of Amino Acids into Protein of Ehrlich Ascites Carcinoma

Experiment No.		Aerobic		Anaerobic			
				Without glucose		With glucose, 0.015 M	
		O_2 uptake*	Incorporation	CO_2 evolved	Incorporation	CO_2 evolved	Incorporation
		μl.	μmoles per gm. protein per hr.	μl.	μmoles per gm. protein per hr.	μl.	μmoles per gm. protein per hr.
1	Leucine†	142	6.8	33	0.0	659	4.9
2	Valine	207	6.2	37	0.0	682	4.5
3	Lysine	167	5.1	33	0.0	651	3.4
3	Phenylalanine	170	2.7	35	0.0	644	1.8
4	Methionine	193	2.0	39	0.0	690	1.2

* Oxidation of endogenous substrate.

† All substrate amino acids were 10^{-3} M. The incubation period was 1 hour.

with oxidation-linked incorporation (see Curve 1, Fig. 3). In addition it is evident from Fig. 1 that the delay in incorporation was not due to an induction period in glycolysis itself. The delay also does not appear to be due to the accumulation of a high energy form of the amino acid. If such were the case, a continuation of incorporation following the end of glycolysis under conditions of low glucose concentration might be anticipated. The induction period appears to be related to the preliminary equilibration period of the cells in the side arm of the Warburg apparatus, where the cells were kept for 10 minutes without substrate. A less marked induction period was also elicited under aerobic conditions by keeping the cells in the side arm for a longer period without adequate oxygenation.

This induction period, which accompanied glycolysis-linked incorporation but not oxidation-linked incorporation under the conditions studied,

XUM

leads to lower values for the glycolysis-linked incorporation, as shown in Table I. Independent experiments demonstrated that optimal rates of incorporation of leucine under both oxidative and glycolytic conditions are equal.

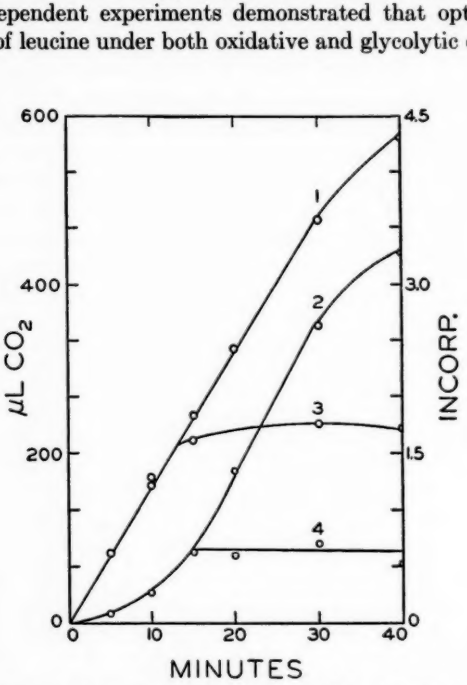


FIG. 1. The effect of glucose concentration upon the rate and duration of anaerobic glycolysis and the rate of incorporation of leucine into protein. Curve 1, anaerobic glycolysis; glucose concentration 30 μmoles in 2 ml. total volume. Curve 2, incorporation coupled to glycolysis of Curve 1. Curve 3, anaerobic glycolysis; glucose concentration 5 μmoles in 2 ml. total volume. Curve 4, incorporation coupled to glycolysis of Curve 3. Incorporation expressed in micromoles of leucine per gm. of protein. Leucine concentration, 1×10^{-3} M.

Nature of DNP Inhibition—In the experiments discussed in the following section, DNP was employed to dissociate oxidation from phosphorylation, thus causing the incorporative process to function under conditions of a suboptimal energy supply. It was therefore necessary to ascertain whether the concentration of inhibitor used could inhibit the incorporative process directly. The following observations are pertinent to this consideration.

When several concentrations of DNP were used in the medium of a system incorporating leucine into protein, the major extent of the inhibition occurred when the oxygen uptake was stimulated (Fig. 2). Identical results were obtained when valine was the amino acid incorporated. Such

a result would be anticipated if DNP released the oxidation-regulating function of phosphorylation (9, 10). The data resemble those obtained by Frantz and coworkers with rat liver slices (11). The inhibition caused by DNP did not resemble the immediate competitive inhibition caused by amino acid analogues (5), but increased with time (Fig. 3) and was non-

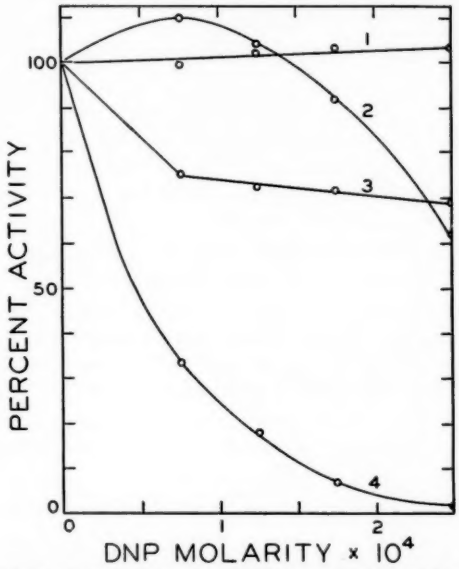


FIG. 2. The effect of dinitrophenol on the incorporation of leucine into protein during glycolysis and during aerobic oxidation. Curve 1, anaerobic glycolysis, glucose concentration, 30 μmoles in 2 ml. total volume; 100 per cent = 710 μl. of carbon dioxide evolved. Curve 2, oxidation; 100 per cent = 208 μl. of oxygen uptake. Curve 3, glycolysis-linked incorporation; 100 per cent = 4.2 μmoles incorporated per gm. of protein. Curve 4, oxidation-linked incorporation; 100 per cent = 7.7 μmoles incorporated per gm. of protein. Time of incubation, 1 hour; leucine concentration, 1×10^{-3} M.

competitive (Fig. 4). An agent which depleted the energy supply of the cell would be expected to produce a non-competitive, gradually increasing inhibition.

Since the formation of high energy phosphate accompanying anaerobic glycolysis is not inhibited by DNP (12, 13), the effect of this inhibitor upon glycolysis-activated amino acid incorporation might be used as a criterion for a possible direct inhibition of the incorporative process. However, even under anaerobic conditions any adenosinetriphosphate (ATP) formed during glycolysis would be in equilibrium with the oxidative phosphorylation

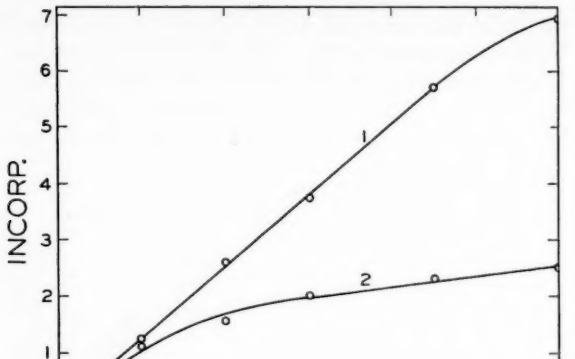


FIG. 3. The effect of time of incubation upon the inhibition by dinitrophenol of leucine incorporation into protein. Curve 1, incorporation rate of aerobic incubation; leucine concentration, 1×10^{-3} M. Curve 2, as above, in the presence of 5×10^{-5} M dinitrophenol. Incorporation expressed in micromoles of leucine per gm. of protein.

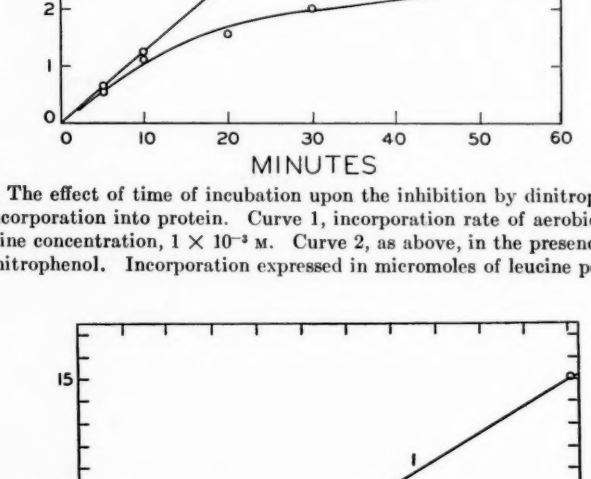


FIG. 4. The effect of a preincubation with dinitrophenol upon the subsequent incorporation of leucine at several concentrations. Curve 1, in the presence of 5×10^{-5} M dinitrophenol. Curve 2, uninhibited oxidation-linked incorporation of leucine. Preincubation with dinitrophenol, 30 minutes; incubation with leucine, 15 minutes. Incorporation expressed in micromoles of leucine incorporated per gm. of protein during the 15 minute incubation period.

mechanism and could therefore be depleted by the DNP-induced hydrolysis of its high energy linkages (14). An inhibition of the glycolysis-linked incorporation of leucine was observed, but its extent in the presence of various concentrations of DNP indicated that it did not directly affect the incorporative process. As the inhibitor concentration was increased, the degree of DNP inhibition of glycolysis-activated leucine incorporation reached a limiting value of 30 per cent, while the inhibition of oxidation-linked incorporation rapidly approached completion (Fig. 2). Such a relationship would be anticipated if the ATP formed during glycolysis could have been dissipated through the DNP-induced breakdown. Under these conditions a limiting rate of high energy phosphate depletion would preclude the complete removal of ATP from the incorporative system. Under oxidative conditions, however, a direct block in the sequence of ATP formation occurred, and none was available to support incorporation at the higher DNP concentrations. No such difference between oxidation and glycolysis-linked incorporation should have been observed if DNP affected the incorporative process directly.

DNP Inhibition of Multiple Amino Acid Incorporation—It was previously suggested (5), on the basis of the extent of mutual inhibition, that leucine and phenylalanine could not be activated at the same enzyme site prior to incorporation. The experiments reported here are designed to ascertain whether the same source of energy can be used for preliminary activation of different amino acids. For this the effect o energy depletion by DNP upon the incorporation of amino acids, both singly and in groups, was determined.

When amino acids were present together in the medium at equal concentrations, they were incorporated into Ehrlich ascites protein independently. Similar results were obtained by Borsook and coworkers (15) with bone marrow cells. However, if amino acids were competing for an energy source limited by DNP, groups of amino acids should no longer be activated independently. One would expect that under these conditions the total quantity activated should remain constant. This would occur if the limiting supply of energy must be distributed between the amino acids in this group. Such a redistribution of energy would seriously interfere with the independent incorporation of amino acids.

The extent of interference of incorporation of one amino acid which would be caused by an additional amino acid under limiting energy conditions should depend upon the nature of the incorporative process itself. Two possibilities may be considered. If incorporation is an exchange process between independently activated amino acids and residues in the protein molecule, then, under limiting energy conditions, the total amount of amino acids incorporated when more than one is added to the medium would be the same as when only one is added. Essentially the redistribu-

XUM

tion in energy should result in a redistribution of the amino acids being incorporated, but the total number of micromoles of all amino acids being incorporated would not be changed. Incorporation may also be considered to be a synthetic process involving the simultaneous incorporation of endogenous amino acids which are in low concentration. In this case, an amino acid added to the medium in high concentration should effectively compete with the former for the limiting energy and result in a yet more pronounced inhibition of incorporation of groups of added amino acids.

TABLE II

Effect of Dinitrophenol upon Incorporation into Protein of Amino Acids When Present Singly and in Groups

Experiment No.		Incorporation, μmoles per gm. protein per hr.	
		In buffer only	With dinitrophenol, 5×10^{-5} M
1	Leucine 10^{-3} M	7.6	4.4
	Valine 10^{-3} M	5.9	3.6
	Leucine 10^{-3} M + valine 10^{-3} M*	14.0	8.1
2	Leucine 5×10^{-3} M	11.2	3.8
	Valine 5×10^{-3} M	7.4	3.0
	Phenylalanine 5×10^{-3} M	3.5	1.3
	Leucine 5×10^{-3} M + valine 5×10^{-3} M + phenylalanine 5×10^{-3} M†	19.8	6.2

* Obtained by summing the values for flasks containing leucine-C^{14} + valine-C^{12} and leucine-C^{12} + valine-C^{14}.

† Obtained in the manner indicated above. The incubation period was 1 hour.

These conjectures were not borne out experimentally, for, when more than one amino acid was incorporated into protein in the presence of DNP, the total amount of amino acid incorporated increased and approached or exceeded the values for the uninhibited system when amino acids were incorporated singly. Representative experiments demonstrating this phenomenon are presented in Table II. From the above reasoning and the results reported in Table II it may be inferred that the energy for incorporation is not distributed to the various amino acids under competitive conditions, but in a manner by which it may be *allocated* to the amino acids which are protein constituents. Such an allocation could occur by activation of sites either on a template (4) or on an enzyme which is specific for incorporation of an amino acid into a peptide. The latter case has been demonstrated by the formation of glutathione from γ-glutamylcysteine and glycine (16). Our observations would appear to preclude preliminary

amino acid activation either as phosphorylated derivatives (2) or by a transpeptidation type mechanism (3).

SUMMARY

The incorporation of radioactive leucine, valine, lysine, phenylalanine, and methionine into the protein of the Ehrlich ascites carcinoma is supported anaerobically under conditions of active glycolysis. An induction period in the glycolysis-linked incorporation is described.

Dinitrophenol inhibits oxidation-linked incorporation almost to completion in concentrations at which it stimulates oxygen uptake. It is a poor inhibitor of glycolysis-linked incorporation. These facts together with others indicate that dinitrophenol does not inhibit the incorporative process directly, but only depletes its required energy supply.

Under conditions of limiting energy supply brought about with dinitrophenol, groups of amino acids are incorporated into protein independently. This is interpreted as an indication that individual amino acids do not compete with each other for activation, but that this activation is allocated to the amino acids which are protein constituents.

BIBLIOGRAPHY

1. Borsook, H., *Advances in Protein Chem.*, **8,** 159 (1953); also in Greenberg, D. M., Chemical pathways of metabolism, New York, **2,** 204–206 (1954).
2. Sheehan, J. C., and Frank, V. S., *J. Am. Chem. Soc.*, **72,** 1312 (1950).
3. Fruton, J. S., Johnston, R. B., and Fried, M., *J. Biol. Chem.*, **190,** 39 (1951).
4. Dounce, A. L., *Enzymologia*, **15,** 251 (1952).
5. Rabinovitz, M., Olson, M. E., and Greenberg, D. M., *J. Biol. Chem.*, **210,** 837 (1954).
6. LePage, G. A., *Cancer Res.*, **13,** 178 (1953).
7. Yanari, S., Snoke, J. E., and Bloch, K., *J. Biol. Chem.*, **201,** 561 (1953).
8. Zamecnik, P. C., and Keller, E. B., *J. Biol. Chem.*, **209,** 337 (1954).
9. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, **195,** 215 (1952).
10. Rabinovitz, M., Stulberg, M. P., and Boyer, P. D., *Science*, **114,** 641 (1951).
11. Frantz, I. D., Jr., Zamecnik, P. C., Reese, J. W., and Stephenson, M. L., *J. Biol. Chem.*, **174,** 773 (1948).
12. Rowsell, E. V., and Greville, G. D., cited by Judah, J. D., *Biochem. J.*, **49,** 271 (1951).
13. Clowes, G. H. A., and Keltch, A. K., *Proc. Soc. Exp. Biol. and Med.*, **77,** 369 (1951).
14. Hunter, F. E., Jr., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1,** 316 (1951).
15. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **186,** 297 (1950).
16. Snoke, J. E., *J. Am. Chem. Soc.*, **75,** 4872 (1953).

XUM

THE DETERMINATION OF URINARY 17-TRIOXYSTEROIDS*

By RAYMOND RIVOIRE,† JANE RIVOIRE, and JEAN POUJOL

(From the Mediterranean Institute of Experimental Medicine, Nice, France)

(Received for publication, May 19, 1954)

The color reaction characteristic of cortisone and related compounds (with the 17,21-dihydroxy-20-keto grouping), which Porter and Silber (1) reported in 1950, has been used by a number of investigators to measure the "cortisone-like" metabolites of urine. To facilitate expression, we propose to give to the methods of analysis utilizing this reaction the term of "determination of 17-trioxysteroids;"[1] this denomination has a descriptive chemical basis, and it is similar to the one universally utilized to designate the steroids determined by Zimmermann's reaction; *i.e.*, the determination of 17-ketosteroids.

As Kinsella, Baggett, and Glick (2), Schneider (3), Cohen (4), and Venning (5) have shown, it is necessary to incubate the urine with β-glucuronidase to hydrolyze conjugated steroids. The 17-trioxysteroids are eliminated for the most part as glucurono conjugates: without hydrolysis, only the small free fraction, amounting to about one-twentieth of the total, was obtained. Hydrolysis with hydrochloric acid is too drastic for these labile substances, as was demonstrated by Heard, Sobel, and Venning (6).

Perhaps it is not so well known that the extraction of corticoids must be carried out with large volumes of solvent, because the partition coefficient between water and chloroform is not very favorable for these compounds, which are slightly soluble in water and not very soluble in chloroform. Baggett, Kinsella, and Doisy (7) have pointed out the difficulty of extraction of all the 17-trioxysteroids with chloroform.

Finally, the original Porter and Silber reaction, excellent with pure steroids, is much less satisfactory with extracts of urine, because brown color formation by the reaction with concentrated sulfuric acid obscures the specific yellow color. To obviate this difficulty, the sulfuric acid has

* This work has been helped by subventions of the French national Caisse of Social Security and of the French National Institute of Hygiene.

† Fulbright Fellow.

[1] The exact chemical name of these compounds is 17,21-dihydroxy-20-ketosteroids. The term 17-trioxysteroid is appropriate, for it takes into account the essential characteristic of the molecule; *i.e.*, the 3 oxygen atoms in the dihydroxyacetone grouping at C-17. Furthermore, it is euphonic, easy to remember, and obviates the use of cumbersome expressions. Almost all of the 17-trioxysteroid is in the form of tetrahydrocortisone. Small quantities of cortisone, Compound F, and tetrahydro Compound F have also been demonstrated.

XUM

been replaced by hydrochloric acid, which gives little color with urinary extracts, but yields a yellow color of approximately the same intensity with pure steroids. As an example, with two identical extracts of 5 ml. of the same urine, the optical density of the blank against distilled water (at 415 mμ) with 4 ml. of the methanol-sulfuric acid reagent was 0.75, and with 4 ml. of the methanol-hydrochloric acid reagent, only 0.15. In some cases, the optical density of the blank is even higher than that found for the test-tube with the methanol-phenylhydrazine-sulfuric acid reagent; that is never seen with the reagent containing hydrochloric acid, even for urine containing a small amount of 17-trioxysteroids, as in Addison's disease. On the other hand, with a sample of 20 γ of pure tetrahydrocortisone, the optical density (at 415 mμ) was 0.17 with 4 ml. of the methanol-phenylhydrazine-sulfuric acid reagent and 0.15 with 4 ml. of the methanol-phenylhydrazine-hydrochloric acid reagent. Without doubt, the hydrochloric acid reagent is much better than the sulfuric acid reagent for the determination of the 17-trioxysteroids in urine, more especially as the stability of the specific color is approximately the same with both reagents (about 30 minutes).

We have established, taking into account these essential points, a very simple and accurate technique, easily utilized in the clinical field, which gives very constant results.

Methods

Reagents—

1. β-Glucuronidase, Sigma.
2. Chloroform, redistilled and washed once with 2 N NaOH and twice with distilled water.
3. Concentrated hydrochloric acid, reagent grade, density 1.19.
4. Phenylhydrazine hydrochloride, reagent grade.
5. Methanol, absolute.
6. Spectrophotometer, Coleman junior.

Hydrolysis—The hydrolysis was conducted by a procedure similar to that described by Baggett *et al.* (7). To 10 ml. of urine, 1000 units of β-glucuronidase and a few drops of chloroform are added, and the pH is adjusted to 6.2 (electrometric). The tube is stoppered and put in the incubator at 37°. After 24 hours, the pH is measured and readjusted to 6.2 if necessary. The tube is put in the incubator for another 24 hours.

Extraction—The urine is acidified to pH 1 with concentrated hydrochloric acid and extracted twice with 50 ml. of chloroform (there is very little or no emulsion). The chloroform extracts are combined and washed once with 10 ml. of 0.1 N NaOH and twice with 10 ml. of distilled water. The chloroform is then put in a beaker in a boiling water bath. When the

volume is reduced to about 25 ml., two equal portions are transferred to test-tubes, and the chloroform is evaporated, preferably under reduced pressure; each tube contains the steroids of 5 ml. of urine.

Reaction—Two reagents are prepared: Reagent A, 0.5 ml. of methanol and 9.5 ml. of concentrated hydrochloric acid; Reagent B, 0.5 ml. of methanol in which are dissolved 6.5 mg. of phenylhydrazine hydrochloride; 9.5 ml. of concentrated hydrochloric acid.

To Test-Tube A, 4 ml. of Reagent A are added; to Test-Tube B, 4 ml. of Reagent B. Both tubes are shaken and transferred to the incubator at 37°. At the same time, the two tubes containing the remainder of Reagents A and B, to be used as blanks, are also transferred to the incubator. After 1 hour, the tubes are cooled, centrifuged 5 minutes, and read at 415 mμ in the spectrophotometer. Test-Tube A is read against the rest of Reagent A; Test-Tube B against the rest of Reagent B. The difference between the optical density of Test-Tube A and B is calculated and the corresponding concentration of 17-trioxysteroids read on a reference curve which had been prepared for tetrahydrocortisone.

Construction of Reference Curve—Two sets of eight tubes are prepared, containing respectively 2, 4, 6, 8, 10, 20, 30, and 40 γ of pure tetrahydrocortisone dissolved in methanol (cortisone or Compound F may also be used for the preparation of standard curves). The methanol is evaporated, and 4 ml. of Reagent A are added to each of one set of tubes; 4 ml. of Reagent B are added to each of the second set of tubes. All the tubes, and also the two tubes containing the rest of Reagents A and B (blanks), are transferred to the incubator. After 1 hour, the tubes of the first set are read against the Reagent A and the tubes of the second set against Reagent B (415 mμ). The true optical density of each sample is calculated by difference, as stated in the preceding paragraph, and the readings plotted on coordinate paper, with optical density on the x axis and weight on the y axis. The curve so constructed is a straight line, corresponding to the Lambert-Beer law, from 10 to 40 γ (up to 80 γ, the curve is in good agreement with the Lambert-Beer law; after 80 γ, some discrepancy is evident). Below 10 γ, the curve is not exactly straight and, of course, the evaluation of results in this area is not so accurate. Since the lower limit of the reaction is 2 γ, 10 ml. of urine must contain at least 4 γ of 17-trioxysteroids for the reaction to be positive. Fortunately, except in some cases of Addison's disease, much larger quantities are found, ranging from 15 to 30 γ in most cases; this gives readings in the most favorable sector of the curve. The color obtained with urinary extracts is a fine golden yellow, very similar to that observed with pure steroids (Fig. 1).

Specificity and Reproducibility of Reaction—The methanol-phenylhydrazine-hydrochloric acid reagent is as specific for the 17-trioxysteroids as the

XUM

original Porter-Silber reagent containing sulfuric acid. Numerous experiments with steroids other than 17-trioxysteroids have yielded uniformly negative results. Perhaps still more convincing are the following experiments. One sample of 10 ml. of hydrolyzed urine is extracted with petroleum ether, which removes the 17-ketosteroids, but not the 17-trioxysteroids. The petroleum ether is washed once with 0.1 N NaOH, twice with distilled water, and divided in two equal parts which are evaporated in two test-tubes. The modified Porter-Silber reaction is carried out on these tubes. In each case, the reaction was entirely negative. The urine is then extracted with 100 ml. of chloroform and the Porter-Silber reaction

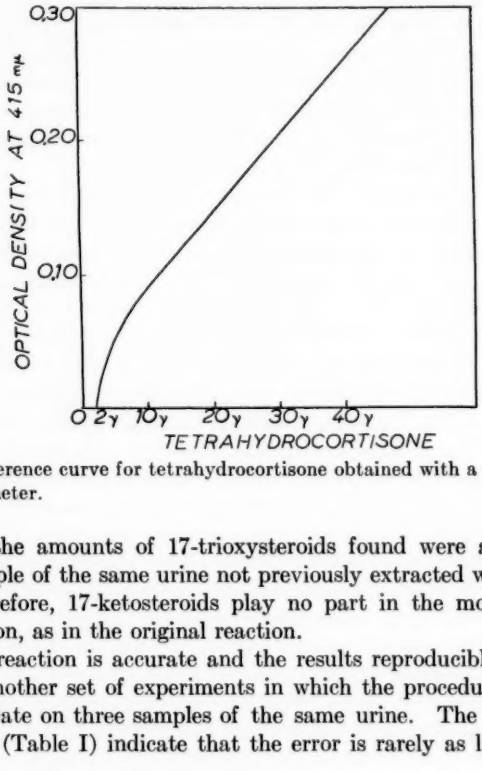


FIG. 1. Reference curve for tetrahydrocortisone obtained with a Coleman junior spectrophotometer.

performed; the amounts of 17-trioxysteroids found were as large as in another sample of the same urine not previously extracted with petroleum ether. Therefore, 17-ketosteroids play no part in the modified Porter-Silber reaction, as in the original reaction.

That the reaction is accurate and the results reproducible was demonstrated in another set of experiments in which the procedure was carried out in triplicate on three samples of the same urine. The results of the experiments (Table I) indicate that the error is rarely as large as 10 per cent.

Recovery of Added Tetrahydrocortisone[2]—In another experiment, pure

[2] The tetrahydrocortisone utilized in these experiments was received from the Worcester Foundation for Experimental Biology, through the generosity of Dr. Gregory Pincus, and from the Roussel Laboratory, Paris.

tetrahydrocortisone was added to 10 ml. of urine having a very low content (0.55 mg. per liter) of 17-trioxysteroids. The tetrahydrocortisone was dissolved in methanol, the desired amount was introduced in a test-tube, and the methanol evaporated. A few drops of 0.1 N NaOH were put into the tube and left in contact for a few seconds; when the dissolution of tetrahydrocortisone was completed, 10 ml. of urine were added. This procedure, as was often verified, is not harmful for tetrahydrocortisone (at least for the results of the Porter-Silber reaction). Each sample was hydrolyzed by glucuronidase, extracted with chloroform, and the modified Porter-Silber reaction performed. In more recent experiments, the tetrahydrocortisone was directly added to the urine in methanolic

TABLE I

*Reproducibility of Results; Analysis of Urine in Triplicate by Procedure Described in This Paper**

Subject	Values for analyses in triplicate		
	γ	γ	γ
I. Normal	19	19	18
II. Normal	21	21	21
III. Normal	18	17	18
IV. Addison's disease	5	5	5
V. Panhypopituitarism	8	7	7
VI. Cortisone	45	43	43
VII. ACTH	70	68	67
VIII. Hibernation	5	5	5
IX. Cortisone	40	40	42
X. "	38	38	37

* All values are for 17-trioxysteroids in 5 ml. of urine.

solution, with similar results. The amounts of 17-trioxysteroids reported in Table II show that the recovery was satisfactory.

Results

The results of the analysis of over 90 urines by the modified procedure are summarized in Table III.

Normal Subjects—The amounts of 17-trioxysteroids excreted are fairly constant, about 6 mg. per 24 hours (a little more for men and somewhat less for women). It seems that this small discrepancy is not related to sex, but due more to a difference in body weights. There exists in fact a rough relationship between the corporal mass and elimination of the 17-trioxysteroids. The lower values for children, 3 mg. per 24 hours, is in accord with this view.

Addison's Disease—The quantities found are much smaller than normal,

but rarely reach the lower limit of sensitivity of the method of determination, except in very severe cases. Administration of ACTH (corticotropin), even intravenously, to these patients does not cause an increased

TABLE II

Recovery of Tetrahydrocortisone Added in Known Amounts to 10 Ml. of Urine

Tetrahydrocortisone added	Duplicate analyses		Recovery of added tetrahydrocortisone	
γ	γ per 10 ml.	γ per 10 ml.	γ	γ
0*	5	5		
10	15	14	10	9
20	24	24	19	19
30	36	34	31	29
40	46	45	41	40
0†	9	10		
10	20	19	10.5	9.5
20	28	28	18.5	18.5
30	39	40	29.5	31.5
40	50	50.5	40.5	41.0

* Tetrahydrocortisone was dissolved in 0.1 N NaOH; urine was added as described in the text.

† A solution of tetrahydrocortisone in methanol was added to urine. The average of the duplicate determinations, 9.5 γ, was used in calculating the recovery.

TABLE III

Elimination of Urinary 17-Trioxysteroids in Normal and Diseased Subjects

Subject	No. tested	Maximum	Minimum	Mean
		mg. per 24 hrs.	mg. per 24 hrs.	mg. per 24 hrs.
Normal men	15	7.2	5	6.15
" women	14	6.8	4.8	5.82
Children, 6 to 11 yrs.	8	5.1	2.5	3.10
Addison's disease	6	3.1	0.37	1.15
Panhypopituitarism	3	3.3	1.4	2.04
Mild pituitary or adrenal deficiency	10	4.5	2.4	3.34
Artificial hibernation	20	2.5	0.4	1.22
ACTH therapy	12	97.2	15.3	35.3
Cortisone therapy, 100 mg. per 24 hrs.	8	27.4	18.1	21.12

excretion of 17-trioxysteroids. Except in subjects whose output indicates moderate adrenal function, it appears that the suprarenal glands in severe cases were unable to react to supplementary stimulation, though intense.

Panhypopituitarism—The figures are also low in this condition, but in

these cases ACTH increased urinary excretion of the 17-trioxysteroids after sufficient medication.

In less severe cases, in which it is difficult to ascertain whether the endocrine defect is pituitary or adrenal, or both, the decrease of the 17-trioxysteroids is moderate. However, it appears that some diminution of these metabolites is not restricted to this disease. As in the case of the 17-ketosteroids, low figures may be encountered in a number of pathological conditions. Accordingly, it is not prudent to conclude that a slight decrease of 17-trioxysteroids always indicates suprarenal insufficiency.

Artificial Hibernation—In artificial hibernation, excretion of 17-trioxysteroids is decreased. Values as low as in very severe cases of Addison's

TABLE IV

17-Trioxysteroids Extractable with Chloroform after Hydrolysis with Glucuronidase and in Butanol Extracts

Subject	Extractable with chloroform after glucuronidase	Butanol extract (free + conjugated trioxysteroids)
	mg. per 24 hrs.	*mg. per 24 hrs.*
I. Normal man	6.15	5.28
II. Normal woman	5.6	5.04
III. Normal man	5.95	5.15
IV. Normal woman	5.72	4.93
V. Addison's disease, man	1.42	0.52
VI. Addison's disease, woman	1.8	0.95
VII. Cortisone therapy, 100 mg. daily intramuscularly	32	26.2

disease have been noted in the subjects in which hypothermia was profound.

We have not had the opportunity to observe cases of spontaneous hypercorticism, but excretion of numerous subjects during treatment with ACTH or cortisone has been studied. The figures were always higher than normal, but varied with the amount injected (especially for cortisone) and with the mode of administration (especially for ACTH). A record elimination of 97 mg. in 24 hours has been found in an asthmatic patient who had received 20 mg. of ACTH by intravenous infusion; however, the same patient eliminated only 9 mg. after the same amount of hormone had been administered in four intramuscular injections (every 6 hours). Patients who received 100 mg. of cortisone intramuscularly daily eliminated about 20 mg. in 24 hours fairly constantly.

DISCUSSION

Our figures for excretion of normal individuals are higher than most of those published previously. The only values similar to ours were reported

by Baggett, Kinsella, and Doisy (7). These authors hydrolyzed conjugates with glucuronidase and separated tetrahydrocortisone by paper chromatography (tetrahydrocortisone represents about four-fifths of the urinary 17-trioxysteroids); they reported between 3 and 6 mg. for the 24 hour excretion. Since these authors have utilized enzymatic hydrolysis, it seems likely that the lower values reported by others are due to incomplete hydrolysis.

Reddy, Forsham, and Jenkins (8) have extracted both free and conjugated trioxysteroids with butyl alcohol and performed the Porter-Silber reaction on their extracts. The results published by them are very similar to ours, varying between 4 and 6 mg. in normal subjects. We have compared Forsham's method and ours, utilizing in both cases the hydrochloric acid variant of the Porter-Silber reaction. In nearly all cases higher values (from 10 to 20 per cent) were obtained for the hydrolyzed sample (Table IV); perhaps the extraction of the 17-trioxysteroid conjugates by butyl alcohol is not complete. However, this sufficiently close agreement between the results of Forsham and ours suggests that nearly all the cortisone-like metabolites of urine are glucuronides of 17-trioxysteroids.

SUMMARY

A very simple method is presented, which allows the determination of the 17-trioxysteroids (cortisone-like steroids) of the urine after hydrolysis by glucuronidase. The clinical application of this technique was stressed.

BIBLIOGRAPHY

1. Porter, C. C., and Silber, R. H., *J. Biol. Chem.*, **185,** 201 (1950).
2. Kinsella, R. A., Jr., Baggett, B., and Glick, J. H., *Federation Proc.*, **10,** 208 (1951).
3. Schneider, J. J., *Federation Proc.*, **10,** 244 (1951).
4. Cohen, S. L., *Federation Proc.*, **10,** 173 (1951).
5. Venning, E. H., Program of Atlantic City meeting of the Endocrine Society, 43 (1951).
6. Heard, R. D. H., Sobel, H., and Venning, E. H., *J. Biol. Chem.*, **165,** 699 (1946).
7. Baggett, B., Kinsella, R. A., Jr., and Doisy, E. A., *J. Biol. Chem.*, **203,** 1013 (1953).
8. Reddy, W., Forsham, P., and Jenkins, D., Program of Chicago meeting of the Endocrine Society, 89 (1952).

DEPRESSION OF SERUM ALKALINE PHOSPHATASE ACTIVITY BY HUMAN SERUM ALBUMIN*

By PHILIP H. HENNEMAN,† G. MARGARET ROURKE,‡ AND W. P. U. JACKSON§

(From the Medical Service and the Chemistry Laboratory of the Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts)

(Received for publication, March 27, 1954)

The daily intravenous administration of 50 gm. of concentrated salt-poor human serum albumin[1] produces a rise in serum albumin level and a decline in urinary calcium and phosphorus excretion (1). These latter changes have been interpreted as evidence for an albumin-induced stimulation of osteogenesis (2). The consistent finding of a concomitant depression of serum alkaline phosphatase activity was thus unexpected, inasmuch as we believe that, in the absence of liver disease, serum alkaline phosphatase is an index of osteoblastic activity.

Fig. 1 illustrates the relation observed between serum alkaline phosphatase and serum albumin levels in eleven experiments on six patients. In general, as the serum albumin level rose, the serum alkaline phosphatase activity decreased 50 to 100 per cent. There was no concomitant constant change in serum globulin levels. The magnitude of depression of serum alkaline phosphatase activity was considerably greater than could be explained by expansion of plasma volume, as judged by decrease in blood hemoglobin concentration and hematocrit.

The experiments *in vitro* which follow were designed to clarify the nature of this unexpected relation between human serum albumin and serum alkaline phosphatase activity.

* The expense of these studies was defrayed by grants to Dr. Fuller Albright from Ayerst, McKenna and Harrison, Ltd., the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and the National Cancer Institute, National Institutes of Health.

† Research Fellow of the Damon Runyon Memorial Fund for Cancer Research, 1950–53. Assistant in Medicine, Massachusetts General Hospital; Instructor, Harvard Medical School.

‡ Research Associate, Department of Medicine, Massachusetts General Hospital; Research Fellow in Medicine, Harvard Medical School.

§ Eli Lilly South African Research Fellow (with Dr. Fuller Albright). Assistant in Medicine, Massachusetts General Hospital and the Harvard Medical School, 1952–53. Present address, Department of Medicine, University of Cape Town, South Africa.

[1] The concentrated human serum albumin used in these studies was generously provided by Dr. Sam T. Gibson of the American Red Cross.

XUM

Methods

Serum alkaline phosphatase was determined by Bodansky's method (3) in which there is a direct proportion between the actual phosphatase concentration (expressed as units) and the mg. of inorganic phosphate liberated

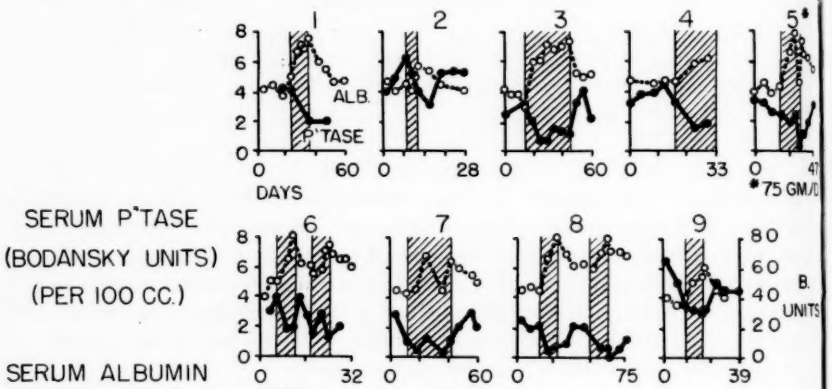


FIG. 1. Effect of daily 50 gm. intravenous albumin administrations on serum albumin levels and serum alkaline phosphatase activity. Nos. 1 to 11 refer to individual experiments. The shaded areas represent the duration of daily albumin infusions. All patients were females except R. E. and K. L. Note the different daily dose of albumin in Experiment 5 and the change in scale of serum alkaline phosphatase in Experiment 9.

from β-glycerophosphate. Thus a mixture of 0.25 cc. of two sera of differing phosphatase content had an activity which was the mean of the two sera tested individually.

Inorganic phosphate was determined by the method of Fiske and Subbarow (4). Albumin and globulin were separated by the Howe technique (5), the Kjeldahl method for nitrogen determination being used.

Albumin and other substances in varying concentration were added to the glycerophosphate substrate in a water bath immediately before the

addition of various "control" human sera of known phosphatase content. None of the substances used altered the pH of the final mixture, which remained at 8.6 when 0.5 cc. of serum was used and at 9.2 when 0.25 cc. of the serum was used. Serum alkaline phosphatase activity was essentially the same at pH 8.6 and pH 9.2. Initially the determinations were made in duplicate, as were all the more important estimations throughout the study. The 25 per cent human serum albumin solutions were free of inorganic phosphate, magnesium, and alkaline phosphatase, and contained 8 to 12 mg. per cent calcium.

Addition of Albumin in Vitro—The addition of 25 per cent commercial serum albumin *in vitro* regularly depressed the alkaline phosphatase activity of various sera. When the first four sera were diluted with an equal volume of albumin solution, an 80 per cent mean depression of alkaline phosphatase activity was achieved: 7.1 to 1.4 units per 100 cc., 8.4 to 1.5, 16.4 to 5.0, and 3.4 to 0.0.

The question then arose whether the effects described above were due to the albumin itself or to its method of preparation with heat and "stabilizers."

Effect of Albumin Stabilizers—Acetyl DL-tryptophanate[2] and sodium-*n*-caprylate[2] are added as stabilizers prior to heating albumin during its preparation for intravenous use. At 0.02 M, the concentration present in commercial albumin solutions, and up to 0.16 M, these substances had no effect on the serum alkaline phosphatase activity.

Effect of "Pure" Albumin—Two samples of unheated dried serum albumin[2] without stabilizers were prepared as 25 per cent solutions. They consistently depressed serum alkaline phosphatase activity whether the latter was originally within the normal range, increased from hepatic or osseous disease, or lowered from previous intravenous albumin administration. As shown in Fig. 2, the degree of depression was roughly proportional to the concentration of albumin present.

Further evidence that this depression of serum alkaline phosphatase activity was an effect of albumin itself is given in Table I. 25 per cent albumin solution was heated to precipitate the albumin. The filtrate was essentially free of depressant effect (Sera I and II). 25 per cent albumin was dialyzed against distilled water for 24 hours, with Visking membrane, and the final contents of the dialysis bag retained the depressant activity of the original albumin solution (Sera III, IV, and VII). Finally 25 per cent albumin was ultrafiltered through a membrane with 180 mm. of Hg-negative pressure. The protein-free ultrafiltrate did not materially depress alkaline phosphatase activity (Sera III, V).

[2] Supplied by Dr. T. D. Gerlough, E. R. Squibb and Sons, New Brunswick, New Jersey.

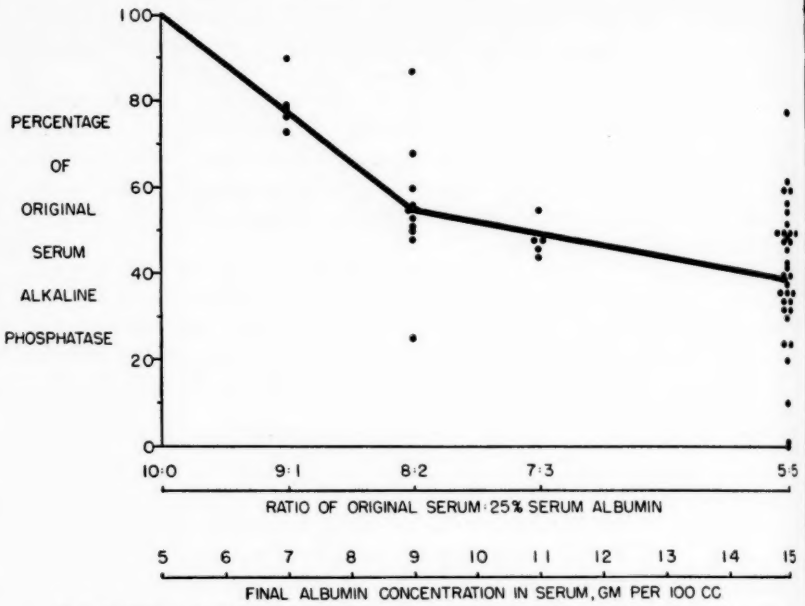


FIG. 2. Effect *in vitro* of human serum albumin on serum alkaline phosphatase activity. The heavy black line indicates the mean of the observations.

TABLE I

Effect in Vitro of Pure Concentrated Serum Albumin, Variously Treated, on Serum Alkaline Phosphatase Activity

	Serum I	Serum II	Serum III	Serum IV	Serum V	Serum VI	Serum VII
Untreated	16.1	7.8	3.8	18.6	12.0	18.8	8.0
Plus 25% albumin 1:1	4.1	3.0		7.8	4.7	6.0	4.1
" 5% " 1:1			1.8		6.1		5.1
" dialyzed albumin 1:1 (equivalent to 8–12% albumin)			3.0	7.0		12.0	5.8
Plus filtrate of heated albumin 1:1	15.5	6.6					
" ultrafiltrate of 25% albumin 1:1			3.0		10.5		

All the values refer to Bodansky units per 100 cc. of serum and are corrected for dilution with the albumin.

Since albumin also depressed serum alkaline phosphatase as measured by the King-Armstrong (6) and Huggins-Talalay (7) methods, this phenomenon does not appear to be specific for a single substrate. Increasing the concentration of substrate (β-glycerophosphate) in the Bodansky

method was also without effect, suggesting that the mechanism of such inhibition was not substrate-binding.

TABLE II

Effect in Vitro of Albumin, Magnesium, and Cobalt on Serum Alkaline Phosphatase Activity

Diagnosis	Untreated serum	+ Mg	+ Co	+ Mg and Co	+ albumin	+ albumin and Mg	+ albumin and Co	+ albumin, Mg, and Co
Paget's disease	23.4	29.8	16.4	19.4	8.2	12.0	8.1	8.2
Liver disease	8.0	11.4			4.1	6.4	4.5	7.0
	9.1	12.0	6.2	6.3	3.4	4.6	4.6	4.3
	12.5				4.5	6.1		8.1
	12.9				6.1	7.7		9.3
	11.1				5.0			9.8
	36.5				15.9			21.7
	22.6				9.4			15.0
	18.0				9.6			9.0
	11.1				5.5			6.7
	11.3				6.7			5.0
	5.8*				2.8			
Bone disease	15.8				6.6			7.0
	80.0				42.0			43.0
Normal†	9.4				3.3			7.5
	5.2				3.1			8.0
	6.5				5.1			4.7
‡	1.1				0.7			
	1.8			1.8	1.2			

All the figures are Bodansky units per 100 cc. of serum.

* Serum Q. See the text.

† Aging of serum accounts for increased phosphatase activity.

‡ The phosphatase activity of these sera (patients K. L. and E. L.) was depressed due to prior albumin therapy *in vitro*.

Rôle of Magnesium and Cobalt—Bodansky (8) has demonstrated that magnesium and cobalt may affect alkaline phosphatase activity. Table II illustrates that 0.01 M magnesium (as $MgCl_2$) may increase the alkaline phosphatase activity of certain sera while 0.001 M cobalt (as $Co(NO_3)_2$) alone or with magnesium may depress the activity of these same sera. Table II also illustrates that magnesium and cobalt, either alone or together, may at least partly reverse the inhibition of alkaline phosphatase activity caused by the addition of albumin to normal and abnormal sera.

XUM

However, sera from patient E. L. (with phosphatase activity depressed to 1.4, 1.0, 0.1, and 1.8 units per cent) were tested with magnesium and cobalt on four occasions, and no raising of the low serum phosphatase whatsoever was found.

Attempts were made to distinguish a rise in serum alkaline phosphatase due to bone disease from that due to liver disease on the basis of the depression caused by albumin or glycine and the restitution of its activity by magnesium, cobalt, or both. No constant differentiating feature was discovered.

Effect of Albumin in Hypoalbuminemic Serum—In the original experiments of this series, depression of alkaline phosphatase activity was associated with hyperalbuminemia. However, as shown in Fig. 1, patient K. L. demonstrated a marked depression of serum alkaline phosphatase activity when his serum albumin was restored from abnormally low to normal values. Indeed, note is made of a slight elevation (5.3 to 5.9 Bodansky units per cent) of serum alkaline phosphatase in this patient prior to treatment while he was hypoalbuminemic (serum albumin 1.86 to 2.06 gm. per cent), and which was not otherwise explained by liver or bone disease. In addition, Table II demonstrates the effect *in vitro* of albumin on the phosphatase activity of Serum Q from a man with hypoalbuminemia due to hepatic cirrhosis. Restitution of his serum albumin level from 0.8 gm. per cent to 3.2 gm. per cent caused a 20 per cent fall in alkaline phosphatase activity; further restitution to 5.6 gm. per cent caused a 50 per cent depression.

These two observations suggest that in certain instances hypoalbuminemia *per se* might be the explanation for an otherwise unexplained modest elevation of the serum alkaline phosphatase.

Experiments with Serum from Patients Receiving Albumin—Sera from patients M. S., K. L., and E. L. (Fig. 1), who were receiving albumin and whose phosphatase activity was already depressed (0.1 to 1.9 units per cent), were mixed with equal volumes of "control" normal and high phosphatase sera. No depression of the control alkaline phosphatase activity was noted in nineteen tests. Presumably the concentration of albumin in the final mixture was not high enough to depress the control serum phosphatase.

SUMMARY

The repeated intravenous administration of human serum albumin raises the recipient's serum albumin level, probably stimulates osteogenesis, but depresses serum alkaline phosphatase activity. Experiments *in vitro* indicate that the *activity* of a given amount of alkaline phosphatase is conditioned by the concentration of albumin present and is markedly depressed by high concentrations of albumin.

BIBLIOGRAPHY

1. Albright, F., Forbes, A. P., Bartter, F. C., Reifenstein, E. C., Jr., Bryant, D., Cox, L. D., and Dempsey, E., Symposia on nutrition, Plasma proteins, Robert Gould Research Foundation, Springfield, **2** (1950).
2. Albright, F., Bartter, F. C., Dempsey, E. F., Forbes, A. P., Henneman, P. H., and Reifenstein, E. C., Jr., in Reifenstein, E. C., Jr., Metabolic interrelations, Josiah Macy, Jr., Foundation, New York, 5th conference (1953).
3. Bodansky, A., *J. Biol. Chem.*, **101,** 93 (1933).
4. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
5. Howe, P. E., *J. Biol. Chem.*, **49,** 93 (1921).
6. King, E. J., and Armstrong, A. R., *Canad. Med. Assn. J.*, **31,** 376 (1934).
7. Huggins, C., and Talalay, P., *J. Biol. Chem.*, **159,** 399 (1945).
8. Bodansky, O., *J. Biol. Chem.*, **115,** 101 (1936); **179,** 81 (1949).

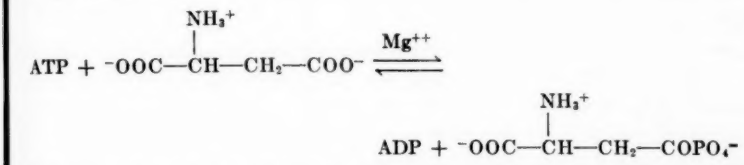
β-ASPARTOKINASE AND β-ASPARTYL PHOSPHATE

By SIMON BLACK and NANCY G. WRIGHT

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Maryland)

(Received for publication, July 26, 1954)

An enzymatic phosphorylation of L-aspartate, which yields β-aspartyl phosphate, was recently found to occur in yeast extracts (1). The reaction is as follows:[1]



(1)

Subsequent work has shown that, in yeast extracts, β-aspartyl phosphate can be enzymatically reduced in two steps to form homoserine (2–5).

The enzyme which catalyzes Reaction 1 will be referred to as β-aspartokinase. A description of some of its properties is presented here with a method for its preparation. A procedure for the chemical synthesis of β-L-aspartyl phosphate and some of its properties is also described.

EXPERIMENTAL

Preparation and Properties of β-L-Aspartyl Phosphate (BAP)

Synthesis[2]—1.5 gm. (4.2 mmoles) of *N*-carbobenzoxy-L-aspartic acid α-benzyl ester, prepared according to Bergmann *et al.* (6),[3] were used in each preparation. It was converted to *N*-carbobenzoxy-L-aspartyl α-benzyl

[1] Abbreviations used are ATP (adenosinetriphosphate), ADP (adenosinediphosphate), AMP (adenosine-5-phosphate), BAP (β-aspartyl phosphate), Tris (tris(hydroxymethyl)methylamine), TPNH (reduced triphosphopyridine nucleotide).

[2] A brief description of this synthesis was previously published (5).

[3] The melting point of carbobenzoxy-L-aspartic acid anhydride, an intermediate in this synthesis, was first reported by these workers to be 84° and later corrected to 124°. We found the L isomer to melt at 109–111°. This discrepancy has very recently been resolved by John and Young (7), who found that the compound can exist in two different crystalline states, one of which melts at 111° and one at 124°. A racemic compound was obtained by using acetic anhydride which analyzed substantially less than 97 per cent for the ring closure; this optically inactive preparation melted at 127°.

XUM

ester β-chloride by the procedure of the same authors. The latter compound was immediately suspended (partially dissolved) in 75 ml. of dry ether and transferred to a 250 ml. Erlenmeyer flask which contained an intimate mixture of 0.6 ml. of H_3PO_4 (85 per cent) plus 1.2 gm. of Ag_3PO_4. The silver phosphate mixture, under the ether solution, was broken into small fragments with a stirring rod. A $CaCl_2$ tube was then attached to the flask and the latter was mechanically shaken for 30 minutes at room temperature. The suspension was briefly centrifuged in capped tubes to give a clear ether solution of *N*-carbobenzoxy-L-aspartyl α-benzyl ester β-phosphate. The yield of this substance was about 80 per cent, as determined by the hydroxamic acid test for acyl phosphate. A qualitative test with $AgNO_3$, after heating a sample with HNO_3, showed the absence of acyl chloride.

Removal of the carbobenzoxy and benzyl groups was achieved by catalytic reduction. The ether solution was transferred to a hydrogenation apparatus (Fig. 1) which was immersed in an ice bath and contained 15 ml. of water, 6.0 ml. of 2 M $KHCO_3$, and 1 gm. of palladium black. Hydrogen was passed through the apparatus at a rate sufficient to mix the ether and aqueous layers vigorously and keep the palladium suspended. This was continued until all of the hydroxamic acid-forming substance in the aqueous solution was reactive in the reverse reaction enzyme test described below. The ether by this time had completely evaporated. The solution was filtered by suction through the sintered disk of the hydrogenation vessel and, if necessary, separated from any toluene not carried away by the hydrogen stream. The pH was[4] about 6.5.

The BAP concentration was 55 μmoles per ml., which indicates an overall yield of 28 per cent. This solution was used in the experiments described here. It was stored at −20°, where it deteriorated slowly but remained useful for several weeks. Attempts to purify the substance further as a silver, lithium, potassium, or barium salt, or on ion exchange columns, have invariably led to excessive destruction. It was possible, however, to remove most of the contaminating inorganic phosphate by slow addition of 1 M $AgNO_3$ while maintaining the pH at 6 to 7 with small additions of KOH.

Chromatographic Tests for Homogeneity of Synthetic BAP—Examination of the synthetic BAP by paper chromatography indicated the presence of only one ninhydrin-reactive substance, aspartic acid. Thus the acyl phosphate was apparently split completely by drying its solution on the chromatograph paper. The hydroxamic acid formed with hydroxylamine was also chromatographed on paper in a number of solvents and detected by

[4] The pH was determined at intervals during the hydrogenation. If it falls below 6.5, it should be brought to 6.5 to 7.0 with small amounts of 5 N KOH.

the method of Stadtman and Barker (8). Only one hydroxamic acid was found. It behaved in all solvents exactly as synthetic β-aspartohydroxamic acid. The hydroxamic acid derived from the enzymatically formed BAP also moved on paper chromatograms in this manner.

Chromatographic Differentiation of α- and β-Aspartohydroxamic Acids—Separation of the α and β isomers of aspartohydroxamic acid was achieved by chromatographing them on Whatman paper No. 1 with a buffer-saturated phenol solution. The buffer used was 0.067 M DL-alanine adjusted to pH 10 with NaOH. By McFarren's procedure (9) these substances were found to have average R_F values of 0.20 and 0.26, respectively.

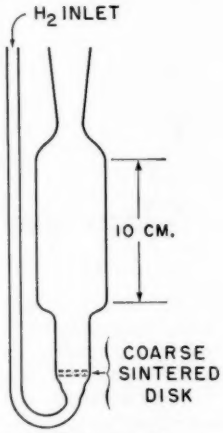


FIG. 1. Hydrogenation vessel

α-Aspartohydroxamic acid was prepared by adding 0.1 ml. of 4 M hydroxylamine hydrochloride to 5 mg. of isoasparagine and heating for 20 minutes at 100°. The product was placed on a small cation exchange column (the hydrogen form of Dowex 50, 50 mesh), washed with water, and the hydroxamic acid eluted with 0.5 M ammonium acetate buffer, pH 5.0.

β-Aspartohydroxamic acid, when treated on chromatograph papers with ninhydrin, produces a brown color such as is formed by asparagine, the β-amide of aspartic acid. The α isomers of the amide and hydroxamic acid produce a typical amino acid blue color.

Effect of pH and Temperature on BAP Stability—Fig. 2 shows the first order hydrolysis rates of BAP at three temperatures and several pH values. The curve representing rates at 30° may be compared to a very similar one reported by Koshland (10) for acetyl phosphate at 30°. The latter shows

sharp upturns in hydrolysis rate at pH 1 and 11, compared to similar upturns with BAP at pH 3 and 13. General stability of BAP is much lower, as its hydrolysis rate at pH 7 (30°) is 3 times the acetyl phosphate rate at this pH and 39°. Despite greater stability in alkaline solution in this experiment, storage at −20° is best at pH 7.

Use of Lowry-Lopez Method with BAP—The relatively fast hydrolysis of BAP in weakly acid solution greatly limits the usefulness of the Lowry-

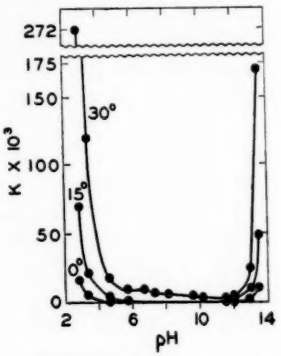


FIG. 2. Hydrolysis rates of BAP. The final concentration of buffer was 0.09 molar in all cases, except at pH 13.5 (approximately), where 0.8 N KOH was used. Buffers employed were sodium sulfate, sodium formate, sodium phthalate, sodium acetate, sodium succinate, imidazole chloride, Tris chloride, ammonium chloride, and sodium carbonate. After the buffer solutions were equilibrated in the constant temperature baths, about 4 μmoles of BAP per ml. were added. BAP was determined at zero time and at three subsequent times. This was done by pipetting 0.5 ml. of the buffered mixture into 0.5 ml. of 2 M hydroxylamine (pH 8), allowing it to stand 10 minutes, and then estimating the hydroxamic acid. The time intervals were chosen whenever possible to give approximately 30, 50, and 70 per cent hydrolysis, but in no case exceeded a total of 6 hours. The rates were first order in all cases. The pH was determined at the end of each experiment with a Beckman model G pH meter with a glass electrode.

Lopez method (11) for inorganic phosphate when BAP is present. This is especially true because small traces of certain proteins cause changes in the breakdown rate of BAP in the presence of the Lowry-Lopez reagents.[5]

[5] In an earlier experiment (1) acyl phosphate values obtained with the hydroxamic acid method were in good agreement with acyl phosphate values calculated from differences in inorganic phosphate found with the Fiske-Subbarow (12) and Lowry-Lopez methods. This agreement was fortuitous, as we have since found both determinations for acyl phosphate, as used then, to give low recoveries with known amounts of synthetic BAP. Hydroxylamine at pH 4.0, used in the first experiment to stop enzyme activity as well as to determine BAP, forms only 60 per cent of the β-aspartohydroxamic acid found when this reagent is used at pH 8.0.

Preparation of β-Aspartokinase

Enzyme Tests—The activity of β-aspartokinase may be determined by measuring the rate of either the forward or the reverse reaction. In the forward reaction test system, the following reagents, other than enzyme,

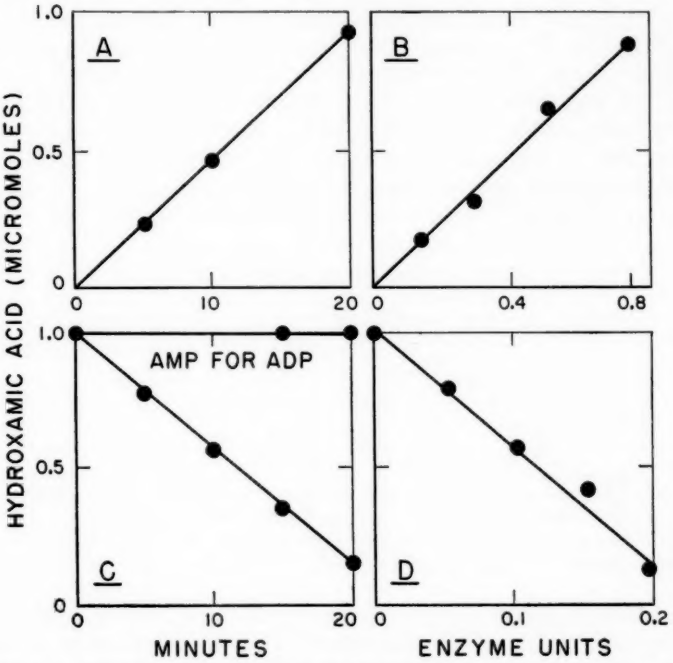


FIG. 3. *A*, Formation of hydroxamic acid in forward reaction enzyme test. 1 unit of β-aspartokinase was used. *B*, relation of enzyme concentration to the forward reaction rate; *C*, BAP utilization in reverse reaction enzyme test (0.2 unit of enzyme was used); *D*, relation of reverse reaction rate to enzyme concentration. The experiments are described in the text.

are used in a final volume of 1 ml. All of these reagents, except $MgCl_2$, are brought to pH 8.0 with Tris base prior to addition: 100 μmoles of L-aspartic acid, 20 μmoles of ATP, 20 μmoles of $MgCl_2$, and 400 μmoles of hydroxylamine hydrochloride. Hydroxylamine reacts with acyl phosphate as it is formed to yield a hydroxamic acid. The latter is determined after a 15 minute incubation at 15°. Crystalline β-aspartohydroxamic acid was used as a standard. 1 unit of enzyme causes the formation of 1 μmole of hydroxamic acid in this test. Fig. 3, *A* and *B* shows that

hydroxamic acid formation is proportional to incubation time and to enzyme concentration.

In the reverse reaction test, final reagent concentrations in micromoles per ml. are 100 Tris chloride buffer (pH 8.0), 10 ADP, 20 $MgCl_2$, and 2 to 4 BAP. The final volume is 0.5 ml. Following a 20 minute incubation at 15°, 0.1 ml. of 0.1 M sodium *p*-chloromercuribenzoate (a suspension) is added to stop the reaction. 5 minutes later 0.4 ml. of 2.0 M hydroxylamine is added. The hydrochloride of the latter is brought to pH 8.0 with Tris base prior to use. After 20 minutes, the hydroxamic acid is determined. Fig. 3, *C* and *D* shows that in this test utilization of BAP is proportional to incubation time and enzyme concentration.

β-Aspartokinase activity can also be demonstrated in an optical test (Fig. 4). When this enzyme system is incubated together with aspartic

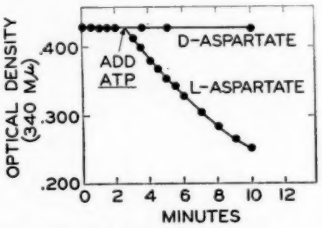


FIG. 4. Optical test for β-aspartokinase. The complete system contained in 1 ml. the following, in micromoles: 100 imidazole chloride buffer (pH 7.0), 0.081 TPNH, 2 $MgCl_2$, 50 L- or D-aspartate, and 4 ATP. Also present were 0.8 unit of β-aspartokinase and 0.9 unit of aspartic β-semialdehyde dehydrogenase.

β-semialdehyde dehydrogenase (2) and TPNH, the BAP formed by the kinase is reduced by the dehydrogenase. TPNH disappearance, observed spectrophotometrically, is a measure of the reduction. The experiment shown illustrates the dependence of the test upon ATP and L-aspartate.

Yeast—6 pounds of fresh Anheuser-Busch bakers' yeast were broken into fine particles by hand and quickly frozen by dropping into liquid N_2, contained in several large beakers.[6] 15 liters of N_2 were required. The frozen yeast was stored in the beakers at −20° until used.

All of the following operations were carried out at 0–5°.

Extraction—250 gm. of the frozen yeast were mixed with 240 ml. of H_2O and warmed to 0°. The pH was then brought to 8.3 to 8.5 with 5 M NH_4OH and the suspension stirred slowly for 2 days. About 300 ml. of extract were obtained by centrifugation at 12,000 × *g*.

Acid and Protamine Treatment—The pH of the extract, now about 7, was

[6] Loss of N_2 by evaporation was minimized by wrapping the beakers in paper.

adjusted to 5.1 with 2 M acetic acid and the inactive precipitate centrifuged. 15 ml. of 2 per cent protamine sulfate were added to the supernatant fluid and the precipitate was discarded after centrifugation.

Ammonium Sulfate Precipitation and Elution—The protamine supernatant fluid was mixed thoroughly with 25 gm. of Whatman cellulose powder ("standard grade"), and 125 ml. of saturated (0°) ammonium sulfate were added slowly with stirring. The suspension was then poured into a 2.4 × 30 cm. glass tube such as is used for chromatograph columns. At the bottom was a coarse sintered glass disk and below this a stop-cock. The supernatant fluid was drained off and the column of precipitated protein and cellulose washed with 25 ml. of 33 per cent saturated ammonium sulfate containing 0.02 M sodium acetate buffer, pH 5.0. The enzyme was

TABLE I

Summary of Enzyme Purification Data

The procedure is described in the text.

Fraction	Volume, ml.	Protein, mg.	Enzyme units	Units per mg.	Yield, per cent
Extract	300	7800	2260	0.29	
Supernatant, pH 5.1	240	4130	1610	0.39	71
Protamine supernatant	252	2612	1600	0.61	71
33% saturated $(NH_4)_2SO_4$ ppt.*		287	1005	3.50	44
Eluate Fractions 25–27	18	7.6	199	26.2	9
" " 28–30	18	36	294	8.2	13

* Data for the 33 per cent saturated $(NH_4)_2SO_4$ precipitate are based on a small scale pilot experiment.

then eluted by the procedure of Zahn and Stahl (13). A mixing chamber above the column contained initially 65 ml. of the 33 per cent saturated ammonium sulfate solution (buffered at pH 5.0). As this solution dripped onto the column, an equivalent volume of 28 per cent saturated ammonium sulfate-0.01 M $KHCO_3$ entered the mixing flask. The flow rate through the column was 30 ml. per hour. Effluent fractions of 6 ml. were collected. Most of the enzyme emerged as a sharp peak in twelve tubes centering about Fraction 28. This effluent was stored at −20° and used in the experiments described here. Table I contains a summary of the purification data.

Properties of β-Aspartokinase Reaction

Metal Activation and Effect of pH—β-Aspartokinase is activated by Mg^{++} ions, which are maximally effective when the concentration is 0.03 M or more (Fig. 5). Mn^{++} ions are more effective at low concentrations, but

reach a maximal activation lower than the Mg^{++} maximum. Fe^{++} ions are partially effective. No activation was found with Pb^{++}, Ni^{++}, Co^{++}, Sn^{++}, Ba^{++}, Ca^{++}, Cu^{++}, Zn^{++}, or Fe^{+++}.

Very little change in the reverse reaction is caused by varying the pH from 5.0 to 9.0. At pH 4.5, complete, irreversible inactivation occurred, and at pH 9.5 the rate was 60 per cent of that observed at pH 5 to 9. A

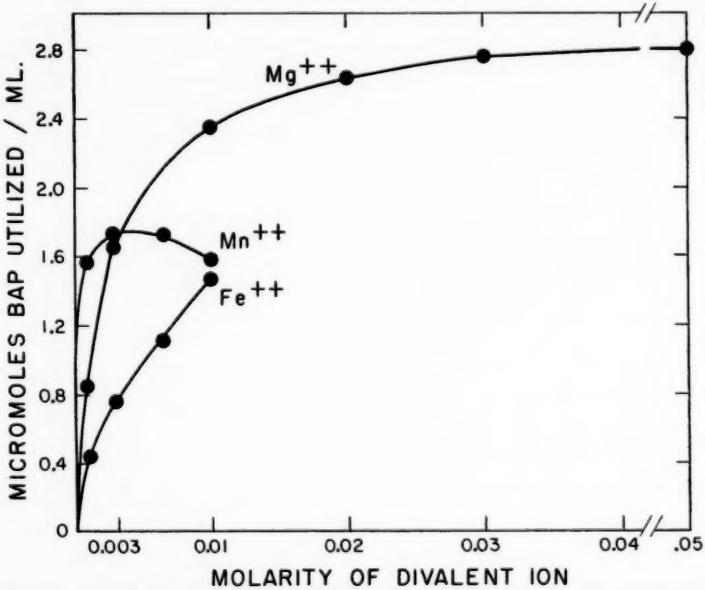


FIG. 5. Activation of β-aspartokinase by divalent metal ions. The conditions of the reverse reaction test system were used in these experiments, except that the incubation time was 12 minutes. Each tube contained 0.16 unit of β-aspartokinase. For each concentration of each metal a control was run without ADP. BAP breakdown due to metal addition was generally very small in the control tests. Mg^{++} and Mn^{++} were added as chlorides and Fe^{++} as $Fe(NH_4)_2(SO_4)_2$.

quite similar relation of the forward reaction rate to pH has also been observed.

Synthesis of ATP in Reverse Reaction—Disappearance of BAP in the presence of ADP and enzyme is accompanied by a stoichiometric formation of ATP, as shown by the experiment recorded in Table II. These data verify the occurrence of the reverse reaction. In control experiments BAP did not disappear in the absence of ADP, and only a very small amount of ATP formed in the absence of BAP.

Equilibrium—The free reversibility of the reaction is illustrated in Fig.

6. With relatively high concentrations of L-aspartate and ATP, BAP accumulated until an equilibrium point was reached. Addition of ADP caused a fall in the BAP concentration to a new equilibrium level. If it is assumed that the appearance of ADP and the disappearance of ATP

TABLE II

Enzymatic Formation of ATP from ADP Plus BAP

The complete system contained initially the following in micromoles per ml.: 50 of imidazole chloride buffer (pH 7.0), 10 of $MgCl_2$, 5 of ADP, 3.2 of BAP, and 0.8 unit of β-aspartokinase. The reaction mixture was incubated for 15 minutes at 15°. The values are in micromoles per ml. of reaction mixture.

	Complete system			−ADP	−BAP
	Initial	Final	Δ	Final	Final
BAP	3.20	0.62	2.58	3.15	
ATP	0.00	2.53	2.53		0.03

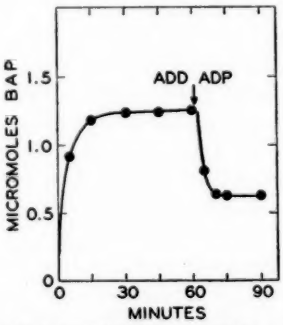


FIG. 6. Equilibrium of the β-aspartokinase reaction. The reaction mixture contained, initially, the following in micromoles per ml.: 250 of L-aspartate, 21.3 of ATP, 20 of $MgCl_2$, and 1.6 units of β-aspartokinase. L-Aspartic acid was brought to pH 8.0 with Tris base prior to use. The incubation was at 15°. 2.86 μmoles of ADP were added in 0.06 ml. at the point indicated. BAP values subsequent to ADP addition are corrected for this dilution. Values plotted are in micromoles per ml.

and aspartate are all equivalent to BAP formation, an equilibrium constant

$$K = \frac{\text{BAP} \times \text{ADP}}{\text{L-aspartate} \times \text{ATP}}$$

can be calculated from the data in Fig. 6. From several experiments such as the one illustrated, an average value of 3.5×10^{-4} has been obtained. This is very close to the value found for an analogous reaction involving ATP and 3-phosphoglyceric acid (14). It must be considered only an

approximation of the true value, under these experimental conditions, because side reactions have not been completely eliminated. The presence of some adenylokinase is indicated by the small formation of ATP from ADP in the absence of BAP (Column 6, Table II).

Specificity—D-Aspartate, L-glutamate, and β-alanine are inactive as substitutes for L-aspartate in the β-aspartokinase reaction. AMP cannot substitute for ADP (Fig. 3, *C*). In joint experiments with Dr. J. L. Strominger, uridine diphosphate, guanosine diphosphate, and inosine diphosphate were also found inactive as substitutes for ADP under the conditions described for the reverse reaction test.

DISCUSSION

Since BAP has not been isolated in a pure state or characterized on the basis of the usual criteria, the evidence for its nature is summarized here. The enzymatically formed substance, treated with hydroxylamine, yielded β-aspartohydroxamic acid which was identified chromatographically. It was quantitatively reduced, enzymatically, to L-aspartic β-semialdehyde (2); the latter was identified and determined by a highly specific enzymatic method (3). A synthetic BAP has been made from a β-aspartyl chloride (with other reactive groups protected) by a method analogous to one used to prepare acetyl phosphate from acetyl chloride (15). The synthetic compound reacts in a manner similar to that of enzymatically formed BAP in the following tests. It yields β-aspartohydroxamic acid with hydroxylamine, it transfers phosphate to ADP in the presence of β-aspartokinase, and it is enzymatically reduced to L-aspartic β-semialdehyde (2).

Subsequent papers (2–3) will describe how BAP is enzymatically reduced in two steps to homoserine, which is an intermediate in the biosynthesis of threonine and methionine (16). Genetic evidence (17) indicates that in *Escherichia coli* threonine and lysine have a common precursor which has not been identified. It is thus possible that BAP is involved, in some species, in the biosynthesis of lysine also. No evidence is available to support an earlier suggestion (1) that BAP is an intermediate in asparagine formation.

Materials and Methods

The sodium salt of ADP was prepared from the barium salt which was obtained from the Sigma Chemical Company. Disodium ATP was obtained from the Pabst Brewing Company. TPNH was prepared by the procedure of Kaplan *et al.* (18). Ag_3PO_4 was made by the method of Lipmann and Tuttle (15). Crystalline β-aspartohydroxamic acid was prepared by a modification of the procedure of Roper and McIlwain (19). Isoasparagine was obtained from Dr. J. P. Greenstein.

BAP and other acyl phosphates were determined by the hydroxamic acid method (20). ATP was determined according to Kornberg (21). Protein was determined by a turbidimetric method (22) when the nucleic acid content was high, and otherwise by the optical method of Warburg and Christian (23). Solvents most useful in paper chromatography of amino acids and derivatives have been water-saturated phenol, *n*-butanol-acetic acid-water (4:1:1), *n*-propanol-water-diethylamine (85:15:4) (24), and methanol-pyridine-water (3:1:1) (24). R_F values for β-aspartohydroxamic acid with these solvents were, respectively, 0.25, 0.09, 0.12, and 0.30.

SUMMARY

1. β-Aspartokinase catalyzes the reversible formation of β-L-aspartyl phosphate (BAP) and ADP from L-aspartate and ATP.

2. This enzyme has been purified about 100-fold from a yeast extract. Purification steps involve precipitation of inactive material with weak acid and protamine. Further fractionation is achieved with ammonium sulfate by the elution method of Zahn and Stahl (13).

3. The enzyme is activated by Mg^{++}, Mn^{++}, or Fe^{++} ions and is equally active at all pH values between 5 and 9.

4. An approximate equilibrium constant for the reaction has been found to be 3.5×10^{-4} at pH 8.0 and 15°.

5. BAP has been synthesized by a reaction between monosilver phosphate and *N*-carbobenzoxy-L-aspartyl α-benzyl ester β-chloride; catalytic hydrogenation in cold $KHCO_3$ solution served to remove the carbobenzoxy and benzyl groups. By chemical and enzymatic tests the synthetic compound is equivalent to the one formed enzymatically.

We are grateful to Dr. J. P. Greenstein and Dr. Alton Meister for valuable advice and assistance in the preparation of BAP.

BIBLIOGRAPHY

1. Black, S., and Gray, N. M., *J. Am. Chem. Soc.*, **75,** 2271 (1953).
2. Black, S., and Wright, N. G., *J. Biol. Chem.*, **213,** 39 (1955).
3. Black, S., and Wright, N. G., *J. Biol. Chem.*, **213,** 51 (1955).
4. Black, S., and Wright, N. G., *Federation Proc.*, **13,** 184 (1954).
5. Black, S., and Wright, N. G., *J. Am. Chem. Soc.*, **75,** 5766 (1953).
6. Bergmann, M., Zervas, L., and Salzmann, L., *Ber. chem. Ges.*, **66 B,** 1288 (1933).
7. John, W. D., and Young, G. T., *J. Chem. Soc.*, 2870 (1954).
8. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **184,** 769 (1950).
9. McFarren, E. F., *Anal. Chem.*, **23,** 168 (1951).
10. Koshland, D. E., Jr., *J. Am. Chem. Soc.*, **74,** 2286 (1952).
11. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162,** 421 (1946).
12. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).

XUM

13. Zahn, R. K., and Stahl, I., *Z. physiol. Chem.*, **293,** 1 (1953).
14. Bücher, T., *Biochim. et biophys. acta*, **1,** 292 (1947).
15. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **153,** 571 (1944).
16. Teas, H. J., Horowitz, N. H., and Fling, M., *J. Biol. Chem.*, **172,** 651 (1948).
17. Davis, B. D., *Nature*, **169,** 537 (1952).
18. Kaplan, N. O., Colowick, S. P., and Neufeld, E. F., *J. Biol. Chem.*, **195,** 107 (1952).
19. Roper, J. A., and McIlwain, H., *Biochem. J.*, **42,** 485 (1948).
20. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159,** 21 (1945).
21. Kornberg, A., *J. Biol. Chem.*, **182,** 779 (1950).
22. Stadtman, E. R., Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **191,** 365 (1951).
23. Warburg, O., and Christian, W., *Biochem. Z.*, **310,** 384 (1941).
24. Redfield, R. R., *Biochim. et biophys. acta*, **10,** 344 (1953).

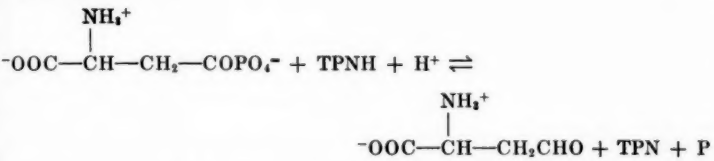
ASPARTIC β-SEMIALDEHYDE DEHYDROGENASE AND ASPARTIC β-SEMIALDEHYDE

By SIMON BLACK and NANCY G. WRIGHT

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Maryland)

(Received for publication, July 26, 1954)

L-Aspartate is converted to L-homoserine in yeast extracts by a series of three enzymatic reactions (1). In the first reaction, described in the preceding paper (2), β-aspartyl phosphate is formed from aspartate and ATP.[1] In the following step β-aspartyl phosphate is reduced to aspartic β-semialdehyde, and the latter, in the third reaction, is reduced to homoserine. The third reaction is discussed in the following paper (3). Evidence is presented here for the first reductive step, which proceeds according to Reaction 1.



(1)

The enzyme which catalyzes Reaction 1 will be referred to as aspartic β-semialdehyde dehydrogenase because of the close analogy between its action and that of 3-phosphoglyceraldehyde dehydrogenase (4). A method for its preparation is described. A method for the synthesis of the D and L isomers of aspartic β-semialdehyde is also presented.

EXPERIMENTAL

Preparation and Properties of Aspartic β-Semialdehyde

*N-Acetyl-*DL*-allylglycine*—20.4 gm. of DL-allylglycine, prepared by Sörensen's method (5), were dissolved in 90 ml. of 2 N NaOH. While the solution was being stirred in an ice bath, 25 ml. of acetic anhydride were added in 5 portions during a 1 hour period. Saturated NaOH, in 3

[1] Abbreviations used are TPN (triphosphopyridine nucleotide), DPN (diphosphopyridine nucleotide), TPNH (reduced triphosphopyridine nucleotide), DPNH (reduced diphosphopyridine nucleotide), P (inorganic orthophosphate), As (arsenate), ADP (adenosinediphosphate), ATP (adenosinetriphosphate), BAP (β-aspartyl phosphate), ASA (aspartic β-semialdehyde), Tris (tris(hydroxymethyl)methylamine), and EDTA (ethylenediaminetetraacetic acid).

ml. quantities, was added to keep the solution above pH 9.5. 50 ml. of concentrated HCl were then added and the solution was extracted twelve times with 500 ml. portions of ethyl acetate. The latter was dried over Na_2SO_4 and evaporated *in vacuo*. *N*-Acetyl-DL-allylglycine crystallized as rectangular plates during the latter stage of the distillation. It was recrystallized from 50 ml. of hot acetone by addition of petroleum ether. The yield was 23.3 gm., 83 per cent of theory; m.p. 114°.

$C_7H_{11}O_3N$. Calculated, C 53.5, H 7.0, N 8.9; found, C 53.6, H 7.0, N 8.7

L-*Allylglycine*—An aqueous solution of 27.8 gm. of *N*-acetyl-DL-allylglycine was brought to pH 7.9 with LiOH in a final volume of 1770 ml. About 10 mg. of hog kidney acylase I (6) were added and the solution incubated at 37° for 15 hours. Hydrolysis was 50 per cent complete, by manometric ninhydrin-CO_2 measurement, in 2 hours; no further hydrolysis occurred. The pH was adjusted to 4.9 with glacial acetic acid, 6 gm. of Norit A were added, and the solution was filtered. On concentrating *in vacuo* to about 100 ml., L-allylglycine began to crystallize in hexagonal plates. Crystallization was completed by addition of 400 ml. of alcohol. The compound was recrystallized from water and alcohol. The yield was 7.1 gm., 70 per cent of theory.

$[\alpha]_D^{24}$ −37.1° (4% in water); −5.7° (2% in 6 N HCl)

$C_5H_9O_2N$. Calculated, C 52.0, H 7.8, N 12.2; found, C 52.0, H 7.9, N 12.3

N-Acetyl-D-*allylglycine*—The alcoholic supernatant solution from the crystallization of L-allylglycine was evaporated to dryness *in vacuo*. The residue was dissolved in 100 ml. of water, brought to pH 0.6 with concentrated HCl, and extracted six times with 250 ml. portions of ethyl acetate. After drying over Na_2SO_4, it was evaporated *in vacuo* to a syrup from which, on cooling, hexagonal plates of *N*-acetyl-D-allylglycine slowly formed. These were dried *in vacuo* and recrystallized three times from hot acetone and petroleum ether. The yield was 9.0 gm., 65 per cent of theory.

$[\alpha]_D^{22}$ −42.0° (4% in alcohol)

$C_7H_{11}O_3N$. Calculated, C 53.5, H 7.0, N 8.9; found, C 53.4, H 7.1, N 9.0

D-*Allylglycine*—A solution of 7.0 gm. of *N*-acetyl-D-allylglycine in 100 ml. of 2 N HCl was boiled for 3 hours under a reflux condenser. After cooling, the pH was adjusted to 5.0 with 4 N LiOH. D-Allylglycine was obtained by concentrating *in vacuo* and crystallizing by addition of alcohol, as with the L isomer. The yield was 3.0 gm., 58 per cent of theory.

$[\alpha]_D^{24}$ +37.8° (4% in water); +5.7 (2% in 6 N HCl)

$C_5H_9O_2N$. Calculated, C 52.2, H 7.8, N 12.2; found, C 51.4, H 7.9, N 12.2

Preparation of L-*Aspartic β-Semialdehyde*—2.3 gm. (20 mmoles) of L-allylglycine were dissolved in 20 ml. of 1 N HCl. Ozone was passed through a fritted glass plug into this solution, held at 0°, for about 100 minutes or 40 minutes beyond the point where ozone absorption was no longer measurable (Fig. 1).

Enzymatic analysis of the solution for L-ASA indicated a yield of 95 per cent of the theoretical amount. A similar experiment with DL-allylglycine yielded 50.3 per cent of L-ASA. The ozonolysis product of D-allylglycine was completely inactive in the enzymatic test.

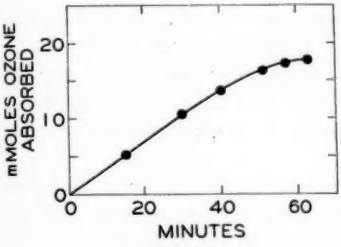


FIG. 1

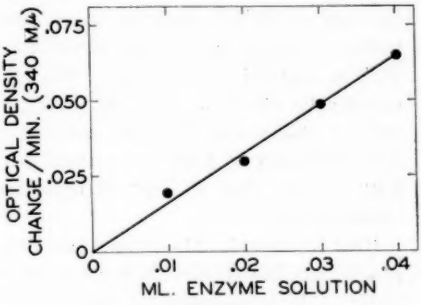


FIG. 2

FIG. 1. Ozone absorption by allylglycine hydrochloride. Ozone was determined by passing it through a solution of KI in boric acid and titrating the liberated I_2 with a standard $Na_2S_2O_3$ solution. Absorption was determined as the difference between influent and effluent O_3. The influent rate, assumed to be constant on the basis of several determinations, was 0.4 mmole of O_3 per minute. Other details are given in the text.

FIG. 2. Relation of reaction rate to enzyme concentration. The procedure is described in the text.

A portion of the ozonolysis product of L-allylglycine containing 6 mmoles of L-ASA was adsorbed on a column of the cation exchange resin Dowex 50 (hydrogen form, 200 to 400 mesh, 2.4 × 14 cm.) and washed with a large volume of water. The aldehyde was then eluted with 4 N HCl. One 6 ml. fraction was collected every 30 minutes. 80 per cent of the adsorbed compound was found in Tubes 6 to 9. This solution was stored in the cold. Immediately before use in enzyme experiments it was diluted to 0.01 M or less and neutralized with $KHCO_3$.

Properties of Aspartic β-Semialdehyde—ASA is reasonably stable in acid solution. It deteriorates markedly in a few hours, however, when neutral, in the dry state as well as in solution. Attempts to dry the hydrochloride by evaporation at low temperatures also resulted in substantial losses of the enzymatically reactive compound.

The usual aldehyde derivatives did not form readily with this substance.

Its identity, therefore, rests largely upon the two enzymatic derivatives, BAP (see below) and homoserine (3). A product which behaves like synthetic ASA on paper chromatograms can also be formed by the enzymatic reduction of BAP or oxidation of homoserine.

Preparation of Aspartic β-Semialdehyde Dehydrogenase

Yeast extract was prepared as previously described (2). Excepting the period when the extract was heated to 60°, the preparation was kept at 0–5° during all operations.

Enzyme Test—The activity of the enzyme was determined by measuring the rate of TPNH oxidation in an optical test. Final reagent concentrations in micromoles per ml. were 100 Tris chloride buffer (pH 8.0), 0.08 TPNH, and 0.4 to 0.6 BAP. The reaction was started after adding the enzyme by the final addition, BAP. The temperature ranged from 23–26°. 1 unit of enzyme is defined as the amount which causes a change in optical density of 1.000 per minute during the 1st minute of the reaction. Fig. 2 shows that the optical density change is proportional to enzyme concentration.

Heat Treatment—The extract was brought to pH 6.5 with 2 M acetic acid. It was then heated rapidly in a boiling water bath, with continuous stirring, to 60°. After maintaining this temperature for 10 minutes, the solution was cooled to 0° in an ice bath, and the inactive precipitate removed by centrifugation.

Acid Precipitation—The pH of the supernatant solution from the heated extract was adjusted to 4.3 by slow addition, with stirring, of 2 M formic acid. The active precipitate was centrifuged at 12,000 × g, and dissolved in 1 M Tris chloride buffer, pH 8.0.

Protamine—The solution of the acid precipitate, 29 ml., was adjusted to pH 6.5 with 2 M acetic acid, and 6 ml. of 2 per cent protamine sulfate were added with stirring. The inactive precipitate was removed by centrifugation.

Ammonium Sulfate—To 39 ml. of protamine supernatant solution 59 ml. of saturated (0°) ammonium sulfate were added. The small precipitate from this 60 per cent saturated solution was centrifuged off; 18.1 gm. of ammonium sulfate were added to the 87 ml. of supernatant solution to bring the saturation to 90 per cent. The active precipitate, after centrifugation, was dissolved in 5 to 10 ml. of 0.01 M $KHCO_3$-HCl buffer, pH 6.5. It was then dialyzed for 6 hours against a large volume of the same buffer containing 0.001 M EDTA.

In Table I is a summary of the purification procedure. The dialyzed ammonium sulfate precipitate, used in all of the experiments reported here, appeared to be completely free of homoserine dehydrogenase (3) and β-aspartokinase (2).

Properties of Enzyme and of Reaction

Substrate Affinity and pH Optima—By the test system described above, the effects of varying BAP and TPNH concentrations on the forward reaction rate were determined. The results are shown in the left portion of Fig. 3. In the right half of the figure, the same results are plotted accord-

TABLE I

Summary of Enzyme Purification Data

The procedure is described in the text.

Fraction	Volume, ml.	Protein, mg.	Enzyme		Yield, per cent
			Units	Units per mg.	
Extract	255	6610	1840	0.3	
Supernatant, 60°	176	510	1090	2.1	59
Ppt., pH 4.3	29	316	1130	3.6	62
Protamine supernatant	39	179	840	4.7	46
Ammonium sulfate ppt., 90%	7	62	615	9.9	33

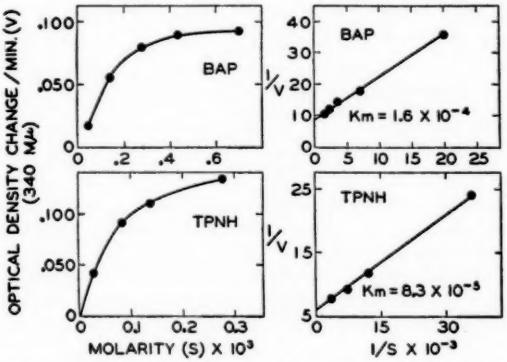


FIG. 3. The relation of substrate concentrations to the forward reaction rate. Except for the variations shown, all concentrations were as indicated for the enzyme test described in the text.

ing to Lineweaver and Burk (7) for the determination of K_m values. The K_m for BAP is 1.6×10^{-4} and for TPNH 8.3×10^{-5}.

The effects of varying substrate concentrations on the rate of the reverse reaction are illustrated in Fig. 4, with the corresponding Lineweaver-Burk graphs. K_m values for ASA, TPN, and P are, respectively, 2.6×10^{-3}, 3.6×10^{-5}, and 1.4×10^{-3}. When arsenate was substituted for P in this test, the rates were about one-half those with corresponding P concentrations.

The forward reaction shows a pH-rate maximum at pH 8.0. One-half the maximal rate occurs at pH 6.9 and 8.6. The reverse reaction is most rapid at pH 9, with 50 per cent of the maximum at pH 8.4 and 9.5. When arsenate is substituted for P in the reverse reaction, the rate is optimal and constant between pH 8 and 9, but falls rapidly on either side of this range.

Stoichiometry—Incubation of synthetic BAP and TPNH with the enzyme results in equivalent decreases of these two compounds and a cor-

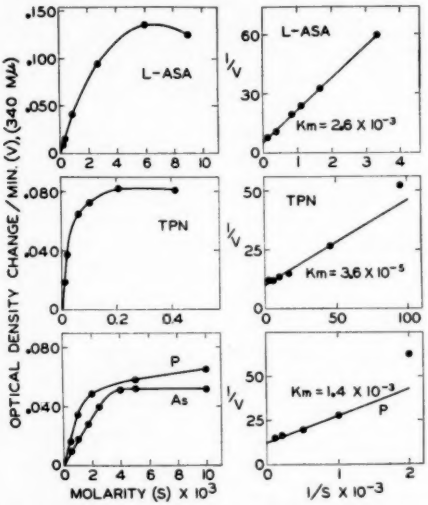


FIG. 4. The relation of substrate concentration to the reverse reaction rate. The complete system contained, per ml., except for the variations shown, 100 μmoles of diethanolamine chloride buffer (pH 9.0), 0.06 μmole of TPN, 10 μmoles of K_2HPO_4, 6 μmoles of L-ASA, and 0.18 unit of ASA dehydrogenase. The final volume was 1.0 ml. contained in a quartz cell at room temperature. The reaction was started by the addition of ASA, and optical density readings were then taken every 30 seconds for 4 minutes.

responding formation of ASA, according to forward Reaction 1 (Table II). BAP formed by the β-aspartokinase reaction was similarly reduced to ASA.

Addition of ASA, TPN, and P to the system results in equivalent formation of BAP and TPNH, and a decrease of ASA according to reverse Reaction 1 (Table II).

Participation of P in Reverse Reaction—Because of the lability of BAP, satisfactory analyses for P in its presence were not possible. Thus the participation of the latter in the reverse reaction could not be shown by demonstrating its utilization. Participation in the reaction was demon-

strated, however, by the dependence of the equilibrium level of TPNH on P concentration (Fig. 5; Table III). The dependence of the reaction on P is also illustrated by the data in Fig. 4.

TABLE II

Stoichiometric Interconversion of BAP and ASA

The complete system contained, in addition to the constituents indicated, per ml., the following: at pH 7.0, 100 μmoles of imidazole chloride buffer and 2.0 units of ASA dehydrogenase; at pH 9.0, 75 μmoles of $KHCO_3$, 20 μmoles of K_2HPO_4, 0.28 μmole of TPN, and 1.8 units of ASA dehydrogenase. The reactions were started by the addition of enzyme and occurred at room temperature. The final volume was 1.2 ml., the reaction time 12 minutes. The values are given in micromoles per ml.

Experiment No.			BAP	TPNH	ASA
1	Synthetic BAP, pH 7.0	Initial	0.27	0.28	0.00
		Final	0.10	0.14	0.14
		Δ	−0.17	−0.14	+0.14
2	β-Aspartokinase-BAP, pH 7.0	Initial	0.34	0.27	0.00
		Final	0.10	0.06	0.21
		Δ	−0.24	−0.21	+0.21
3	As (*1*); DPNH for TPNH	Initial	0.27	0.28*	0.00
		Final	0.25	0.27*	0.00
		Δ	−0.02	−0.01*	0.00
4	Reverse reaction, pH 9.0	Initial	0.00	0.00	0.30
		Final	0.16	0.16	0.15
		Δ	+0.16	+0.16	−0.15

* DPNH values.

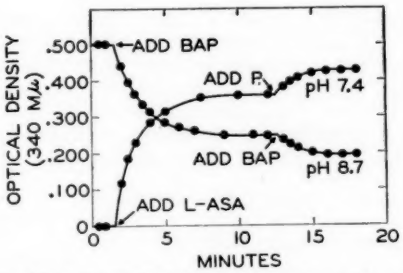


FIG. 5. Equilibrium levels of TPNH found in the forward and reverse reactions. The experimental details are given in Table III.

Specificity—In the forward reaction experiment summarized in Table II, DPNH could not be substituted for TPNH. DPN and D-ASA were completely inactive in the reverse reaction. Though BAP is rapidly arseno-

lyzed by the enzyme (see below), acetyl phosphate is not affected by a high enzyme concentration in the presence of arsenate.

Identification of Products of Forward and Reverse Reactions—The product of BAP reduction has been identified and determined quantitatively as L-ASA by its reactivity with DPNH in the highly specific homoserine dehydrogenase system (Table II).

BAP formed by ASA oxidation has similarly been identified by its reactivity with ADP in the β-aspartokinase system. For this purpose BAP

TABLE III

Equilibrium Concentrations of Reactants

The forward reaction system contained, per ml., in addition to the constituents indicated in Lines 1 and 3, 100 μmoles of Na borate buffer (final pH 8.7) and 1.8 units of ASA dehydrogenase. BAP, containing P, was added at the points indicated in Fig. 5. The reverse reaction system contained, per ml., in addition to the constituents shown in Lines 5 and 7, 100 μmoles of imidazole chloride buffer (final pH 7.4) and 1.8 units of ASA dehydrogenase. ASA and K phosphate (pH 7.4) were added at the points indicated in Fig. 5. The volume for Lines 1, 2, 5, and 6 was 1 ml., and for Lines 3, 4, 7, and 8 was 1.01 ml. The solutions were all at room temperature. The pH at the end of each experiment was determined with a Beckman model G pH meter with a glass electrode. The final concentration values were calculated from TPNH determinations (shown in Fig. 5) as described in the text. All the values are expressed as micromoles or microequivalents per ml.

Line No.		BAP	TPNH	$H^+ \times 10^8$	ASA	TPN	P	$K \times 10^{-4}$
1	Initial	0.114	0.081		0.000	0.000	8.89	
2	Final	0.073	0.040	2	0.041	0.041	8.93	2.6
3	Initial	0.228	0.081		0.000	0.000	11.77	
4	Final	0.179	0.032	2	0.049	0.049	11.82	2.5
5	Initial	0.000	0.000		2.40	0.124	3.00	
6	Final	0.059	0.059	40	2.34	0.065	2.94	3.2
7	Initial	0.000	0.000		2.40	0.124	6.00	
8	Final	0.071	0.071	40	2.33	0.053	5.93	3.6

was accumulated in a dismutation reaction involving ASA dehydrogenase and homoserine dehydrogenase. The latter reaction,

$$\text{ASA} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{homoserine} + \text{TPN} \quad (2)$$

coupled with Reaction 1, gives Reaction 3:

$$2\text{ASA} + \text{P} \rightleftharpoons \text{homoserine} + \text{BAP} \quad (3)$$

BAP accumulation in such a combined system is shown in Fig. 6. Its formation is dependent on ASA and ASA dehydrogenase. At the indicated point, β-aspartokinase, ADP, and $MgCl_2$ were added, resulting in a rapid disappearance of BAP. In control tests omission of ADP or β-aspartokinase prevented this disappearance.

Equilibrium—Reaction 1 can come to an equilibrium position starting with BAP and TPNH or with ASA, TPN, and P. A determination of the

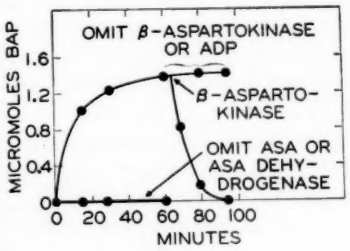


FIG. 6

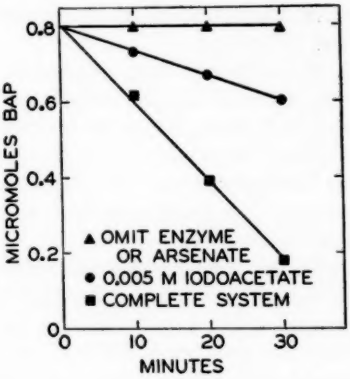


FIG. 7

FIG. 6. Formation of BAP by ASA dehydrogenase and its reactivity with the β-aspartokinase system. The complete system contained initially, per ml., 100 μmoles of diethanolamine chloride buffer (pH 9.0), 0.21 μmole of TPN, 20 μmoles of K_2HPO_4, 6 μmoles of ASA, 0.73 TPNH unit of homoserine dehydrogenase, and 0.9 unit of ASA dehydrogenase. The initial volume was 3.0 ml. and the incubation temperature 15°. At the point indicated for β-aspartokinase addition the following were added, per ml., to the complete system: 4 μmoles of ADP, 8.4 μmoles of $MgCl_2$, and 0.8 unit of β-aspartokinase. BAP determinations were made on 0.2 ml. aliquots of the reaction mixture. The values shown are in micromoles per ml.

FIG. 7. Arsenolysis of BAP in the presence of ASA dehydrogenase. The complete system contained, per ml., 100 μmoles of Tris chloride buffer (pH 8.0), 20 μmoles of Na_2HAsO_4, 1.6 μmoles of BAP, and 0.14 unit of ASA dehydrogenase. The initial volume was 2.5 ml. and the incubation temperature 15°. BAP was determined on 0.5 ml. aliquots of the reaction mixture, for which values are shown. Prior to the addition of hydroxylamine for the BAP determination, each aliquot was incubated 5 minutes with 0.1 ml. of 0.1 M Na *p*-chloromercuribenzoate (a suspension).

equilibrium constant

$$K = \frac{\text{ASA} \times \text{TPN} \times \text{P}}{\text{BAP} \times \text{TPNH} \times \text{H}^+}$$

has been made, as described in the following paragraphs, from the experiments recorded in Fig. 5 and Table III.

In the forward reaction it was assumed that, in approaching equilibrium, the decrease in TPNH concentration is equivalent to the BAP decrease and to the amount of ASA, TPN, and P formed. This assumption is supported by the stoichiometry demonstrated in Table II. Similarly, in the reverse reaction TPNH formation was assumed to be equivalent to BAP

synthesis and to ASA, TPN, and P utilization. The P concentration at equilibrium was considered to be equal to the analytical value by the Fiske-Subbarow method (8) minus the calculated amount of BAP. The H^+ ion concentration, in equivalents per ml., was calculated from determined pH values.

Table III shows that, at the two equilibrium levels of TPNH attained in the forward reaction experiment at pH 8.7, illustrated in Fig. 5, K values of 2.6 and 2.5×10^6 were found. In the reverse reaction, at pH 7.4, values of 3.2 and 3.6×10^6 were obtained. It is possible that complete equilibrium was not quite achieved from either direction, and that the best approximation of the true value is an average of the above, about 3.0×10^6.

Arsenolysis of BAP and Inhibition by Iodoacetate—3-Phosphoglyceraldehyde dehydrogenase catalyzes a splitting of acyl phosphate in the presence of arsenate, a reaction which has been shown to require DPN and is inhibited by iodoacetate (9–13). ASA dehydrogenase similarly causes an arsenolysis of BAP which is inhibited by iodoacetate (Fig. 7). This inhibition is paralleled by an inhibition of the reduction of BAP. At iodoacetate concentrations of 10^{-2} M and 10^{-3} M, both processes are inhibited about 95 and 10 per cent, respectively.

In contrast to 3-phosphoglyceraldehyde dehydrogenase, however, no involvement of a pyridine nucleotide could be demonstrated in the arsenolytic reaction. Treatment of the enzyme with adsorbent charcoal by the procedure of Taylor *et al.* (14) for removing DPN did not affect the reaction. Treatment with the anion exchange resin, Dowex 1 chloride, by a procedure used to remove coenzyme A from enzyme preparations (15) was also without effect. A final determination of the rôle of TPN in this reaction must await a more certain method for obtaining a TPN-free enzyme.

DISCUSSION

There is a close similarity between this reaction and the one catalyzed by 3-phosphoglyceraldehyde dehydrogenase (4). In each case an aldehyde, in the presence of P, is reversibly oxidized to an acyl phosphate by a pyridine nucleotide. It is therefore of interest to compare their known properties. Both reactions are inhibited by iodoacetate, though ASA dehydrogenase is considerably less sensitive to this reagent. Each enzyme catalyzes an arsenolysis of its acyl phosphate substrate, and in both cases the arsenolysis is also inhibited by iodoacetate. The equilibrium constants differ by a factor of 10. A constant of 3×10^7 for the 3-phosphoglyceraldehyde dehydrogenase reaction was calculated from data given by Bücher (16) and by Meyerhof and Oesper (17), using an equation analogous to the one used here. A dependence of arsenolysis on pyridine nucleotide, such as has been

shown for 3-phosphoglyceraldehyde dehydrogenase (9–10), was not apparent with aspartic β-semialdehyde dehydrogenase. It is not certain, however, that the latter enzyme was free of nucleotide when tested.

Materials and Methods

TPN (80 per cent pure), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and DPNH were obtained from the Sigma Chemical Company. TPNH was prepared by the method of Kaplan *et al.* (18). Barium ADP was obtained from the Sigma Chemical Company and converted to the sodium salt. β-Aspartokinase and chemically synthesized BAP were prepared as described separately (2), as was also homoserine dehydrogenase (3). BAP formed by β-aspartokinase was accumulated in an experiment identical with the one described in the preceding paper (2, Fig. 6). When equilibrium was reached, a small aliquot of the mixture was used directly in the experiment described here. β-Aspartokinase activity was effectively stopped by dilution.

All experiments involving measurement of TPNH or DPNH by light absorption at 340 mμ were performed with quartz cells (Pyrocell) having inside dimensions of 5 × 10 × 38 mm. The light path was 10 mm. The volume was usually 1 ml. Both compounds were assumed to have an extinction coefficient of 6.22×10^6 sq. cm. per mole (19). Manometric ninhydrin-CO_2 measurements of amino acids were made by the method of Hamilton and Van Slyke (20). Protein was determined by the optical method of Warburg and Christian (21). BAP was determined by the hydroxamic acid method (22). In the latter case hydroxylamine was found to stop effectively aspartic β-semialdehyde dehydrogenase action; hence there was no formation of BAP in its presence and only BAP formed prior to hydroxylamine addition was measured. L-ASA was determined enzymatically by using homoserine dehydrogenase (3). DPN was determined enzymatically according to Racker (23) and TPN by a similar procedure with glucose-6-phosphate dehydrogenase (24).

SUMMARY

1. Aspartic β-semialdehyde dehydrogenase catalyzes the reversible reduction of β-aspartyl phosphate (BAP) by TPNH to form aspartic β-semialdehyde (ASA) and inorganic phosphate.

2. This enzyme has been purified about 30-fold from an extract of yeast with heat and protamine to precipitate inactive substances, and acid and ammonium sulfate to precipitate the enzyme.

3. Aspartic β-semialdehyde hydrochloride has been synthesized by the ozonolysis of allylglycine hydrochloride in cold aqueous solution. DL-Allylglycine was resolved into its D and L isomers by asymmetric enzymatic

hydrolysis of its *N*-acetyl derivative, and the corresponding isomers of aspartic β-semialdehyde were prepared from them. Only the L isomer was enzymatically active.

4. K_m values for the enzyme were found to be as follows: BAP, 1.6×10^{-4}; TPNH, 8.3×10^{-5}; ASA, 2.6×10^{-3}; TPN, 3.6×10^{-5}; inorganic phosphate, 1.4×10^{-3}. The pH-rate maximum of the forward reaction is at pH 8.0, and for the reverse reaction at pH 9.0.

5. The equilibrium constant for the reaction has been determined to be about 3.0×10^6.

6. The enzyme also catalyzes a splitting of BAP in the presence of arsenate. This arsenolysis, as well as the reduction of BAP by TPNH, is inhibited by iodoacetate.

We are grateful to Dr. S. M. Birnbaum and Dr. J. P. Greenstein for a donation of the acylase enzyme, for the ninhydrin CO_2 analyses, and for other assistance and advice during the preparation of ASA. We are also indebted to Mr. T. D. Perrine and Dr. E. L. May for the use of an ozone generator and the benefit of their experience with this reagent. All microanalyses were performed under the direction of Dr. W. C. Alford.

BIBLIOGRAPHY

1. Black, S., and Wright, N. G., *Federation Proc.*, **13,** 184 (1954).
2. Black, S., and Wright, N. G., *J. Biol. Chem.*, **213,** 27 (1955).
3. Black, S., and Wright, N. G., *J. Biol. Chem.*, **213,** 51 (1955).
4. Warburg, O., and Christian, W., *Biochem. Z.*, **303,** 40 (1939).
5. Sörensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **6,** 187 (1903–06).
6. Birnbaum, S. M., Levintow, L., Kingsley, R. B., and Greenstein, J. P., *J. Biol. Chem.*, **194,** 455 (1952).
7. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
9. Harting, J., and Velick, S., *Federation Proc.*, **11,** 226 (1952).
10. Harting, J., and Velick, S. F., *J. Biol. Chem.*, **207,** 867 (1954).
11. Racker, E., and Krimsky, I., *J. Biol. Chem.*, **198,** 731 (1952).
12. Oesper, P., *J. Biol. Chem.*, **207,** 421 (1954).
13. Racker, E., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, 465 (1954).
14. Taylor, J. F., Velick, S. F., Cori, G. T., Cori, C. F., and Slein, M. W., *J. Biol. Chem.*, **173,** 619 (1948).
15. Stadtman, E. R., Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **191,** 365 (1951).
16. Bücher, T., *Biochim. et biophys. acta*, **1,** 292 (1947).
17. Meyerhof, O., and Oesper, P., *J. Biol. Chem.*, **170,** 1 (1947).
18. Kaplan, N. O., Colowick, S. P., and Neufeld, E. F., *J. Biol. Chem.*, **195,** 107 (1952).
19. Horecker, B. L., and Kornberg, A., *J. Biol. Chem.*, **175,** 385 (1948).
20. Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, **150,** 231 (1943).
21. Warburg, O., and Christian, W., *Biochem. Z.*, **310,** 384 (1941).
22. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159,** 21 (1945).
23. Racker, E., *J. Biol. Chem.*, **184,** 313 (1950).
24. Warburg, O., and Christian, W., *Biochem. Z.*, **292,** 287 (1937).

HOMOSERINE DEHYDROGENASE

By SIMON BLACK and NANCY G. WRIGHT

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Maryland)

(Received for publication, July 26, 1954)

Homoserine arises from L-aspartate in yeast extracts through three enzymatic steps (1, 2). Aspartate reacts with ATP[1] to form β-aspartyl phosphate (3, 4), which is then reduced to aspartic β-semialdehyde (5). In the third step aspartic β-semialdehyde is converted to homoserine, a reaction involving the reversible reduction of an aldehyde group to a primary hydroxyl group.

$$^{-}OOC{-}\underset{\displaystyle NH_3^+}{\underset{|}{CH}}{-}CH_2CHO + DPNH \quad \text{or} \quad TPNH + H^+ \rightleftharpoons {}^{-}OOC{-}\underset{\displaystyle NH_3^+}{\underset{|}{CH}}{-}CH_2{-}CH_2OH + DPN \quad \text{or} \quad TPN \tag{1}$$

The enzyme which catalyzes this reaction will be called homoserine dehydrogenase. A method for its preparation is presented in this paper with a description of some of its properties.

EXPERIMENTAL

Preparation of Homoserine Dehydrogenase

Enzyme Test—The activity of the enzyme was determined in an optical test by measuring the rate of DPNH or TPNH oxidation. The test was made at room temperature in a 1 ml. volume. In addition to enzyme the spectrophotometer cell contained 100 μmoles of potassium phosphate buffer (pH 6.7), and approximately 0.08 μmole of DPNH or TPNH. The reaction was started by the addition of 1.0 μmole of ASA, and optical density readings were then taken every 30 seconds for several minutes. 1 enzyme unit for each pyridine nucleotide is defined as the amount which can cause an optical density change of 1.000 per minute during the 1st minute after

[1] Abbreviations used are ATP (adenosinetriphosphate), ASA (aspartic β-semialdehyde), DPN and TPN (di- and triphosphopyridine nucleotide), DPNH and TPNH (reduced di- and triphosphopyridine nucleotide), Tris (tris(hydroxymethyl)methylamine), EDTA (ethylenediaminetetraacetic acid), and P (inorganic orthophosphate).

ASA addition. The proportional relation of the reaction rate to enzyme concentration, with DPNH or TPNH, is shown in Fig. 1.

Heat Treatment—125 ml. of yeast extract, obtained as described previously (4), were adjusted to pH 6.5 with 2 M acetic acid. The extract, with constant stirring, was then heated to 55° in a hot water bath and maintained at this temperature for 10 minutes. After cooling to 0° in an ice bath the inactive precipitate was removed by centrifugation.

Acid Treatment—The cold supernatant solution from the heated extract was brought to pH 4.6 by slow addition of 2 M formic acid, and the precipitate immediately removed by centrifugation at 0°.

Ammonium Sulfate Precipitation—This step produces no further purification relative to protein but concentrates the enzyme in a small volume and removes most of the nucleic acid.

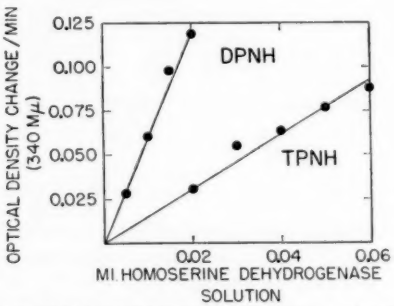


FIG. 1. The relation of the reaction rate to enzyme concentration. The experiment is described in the text.

Without delay the supernatant solution from the acidified solution described above was mixed with 0.82 volume of cold saturated ammonium sulfate solution and the small precipitate immediately centrifuged. 0.375 volume of the saturated ammonium sulfate solution was then added to the supernatant solution to precipitate the enzyme. After centrifugation in the cold, the enzyme was dissolved in 3 to 5 ml. of cold 0.01 M $KHCO_3$-HCl buffer (pH 6.5) containing 0.001 M EDTA, and dialyzed for 15 hours against 2 liters of the same solution.

Chromatography on Calcium Phosphate Column—A chromatographic column, 2.4 × 9.0 cm., was prepared with a mixture of calcium phosphate gel and cellulose powder (6, 7). A portion of the dialyzed solution of the ammonium sulfate precipitate containing 22 mg. of protein was diluted to 15 ml. with 0.01 M potassium phosphate (pH 6.4) and adsorbed on the column at room temperature. 10 ml. of the same potassium phosphate solution were layered over the column, and 40 ml. were placed in a magnetically stirred mixing flask which led to the top of the column through a narrow

tube. As fluid was passed through the column from the mixing flask, an equivalent volume of 0.2 M K_2HPO_4 flowed into the latter from a reservoir. One 2 ml. fraction of eluate was collected every 4 minutes. Large amounts of inactive protein appeared in Fractions 34 to 50 and 54 to 58. Some of the enzyme emerged prior to and following the peak Fractions 66 to 69. Total recovery of activity was about 75 per cent.

A summary of the purification data is given in Table I.

0.5 per cent of crystalline bovine serum albumin was added to the dilute enzyme solution to minimize denaturation. It has been preserved in a frozen state without detectable loss of activity for several months.

TABLE I

Summary of Enzyme Purification

The procedure is described in the text.

Fraction	Volume	Protein	Enzyme, units		Ratio DPNH-TPNH	Units per mg. (DPNH)	Recovery
			DPNH	TPNH			
	ml.	*mg.*					*per cent*
Extract	50	1300	12,800	5280	2.5	10	
Supernatant, 55°	39	564	7,260	2770	2.6	13	57
" pH 4.6	37	126	6,590	2330	2.8	52	52
Ammonium sulfate ppt., 60%	4.6	120	5,940	2210	2.7	50	46
Eluate Fractions 66–69	8	0.43*	520*	168*	3.1	1160	22

* These values must be multiplied by 5.4 to be compared with data from other fractions.

Properties of Enzyme and of Reaction

Effects of Substrate Concentrations and pH on Reaction Rate—The effects on the forward reaction rate of varying the concentrations of ASA and pyridine nucleotides are shown in the left portion of Fig. 2. In the right half of this figure the same data are plotted according to Lineweaver and Burk (8) for the determination of K_m values. The latter are shown, for each substance, in Fig. 2. Corresponding data for the reverse reaction are shown in Fig. 3. Notable in these data is the 7-fold difference in the K_m values for homoserine found when this substrate was tested with the two nucleotides, TPN and DPN. Of the two values for homoserine, the larger was obtained with the pyridine nucleotide, TPN, having the higher K_m value. The TPN K_m is 20-fold larger than the DPN value. The enzyme shows little difference in its affinity for TPNH and DPNH; the reaction partner of these substances, ASA, has similar K_m values when tested with the two reduced nucleotides.

TPNH and DPNH are oxidized in this enzyme system in the same pH

range. The optimal pH for the TPNH reaction is about 5.0 and for the DPNH reaction about 6.8 (Fig. 4).

Activity of Enzyme with Two Pyridine Nucleotides—The ratio of the enzyme's activity with DPNH to activity with TPNH did not change significantly when it was purified over 100-fold (Table I). Destruction of the enzyme by heat was parallel for the two activities, and they were equally protected from heat denaturation by phosphate (Table II).

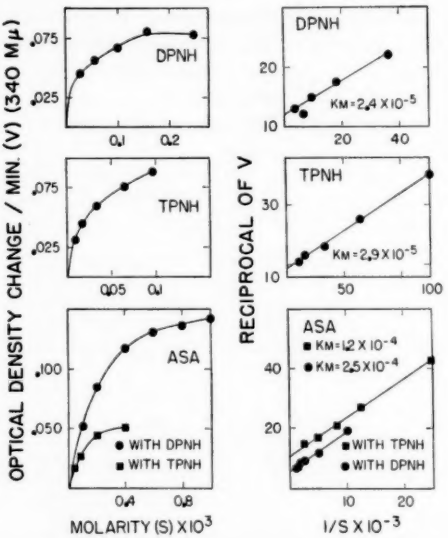


FIG. 2. Relation of the forward reaction rate to substrate concentrations. The conditions described in the text for the enzyme test were used except for the variations in concentration which are indicated.

When TPN and DPN were both added to the system, the rate of reduction by homoserine approximated the rate found with DPN alone (Table III). This was found when the nucleotides were added in a wide range of concentrations, and with large changes in the ratios of their concentrations. With less than the amount of DPN required to half saturate the enzyme, 0.016 μmole, the rate was increased about 20 per cent by the addition of 0.36 μmole of TPN. Experiments with TPNH and DPNH together gave results similar to those obtained in the reverse reaction; *e.g.*, there was no summation of the individual rates of oxidation.

Specificity—Specificity of this enzyme for the L isomers of homoserine and ASA is indicated by the experiment shown in Fig. 5. D-Homoserine does not reduce DPN or TPN, and D-ASA is inactive as an oxidant of

DPNH or TPNH. The carboxyl and amino groups of L-homoserine both appear to be essential for reactivity, as 3-aminopropanol and γ-hydroxybutyrate are completely inert in the system. Also inactive are acetaldehyde (Fig. 5), DL-threonine, DL-serine, and DL-homocysteine.

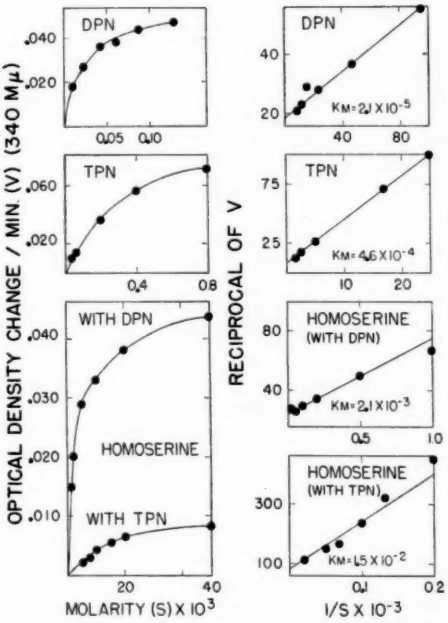


FIG. 3. Relation of the reverse reaction rate to substrate concentrations. The tests were made at room temperature in a volume of 1.0 ml. Except for the variations shown, the system contained 100 μmoles of diethanolamine buffer (pH 9.0), and 0.08 μmole of DPN or TPN. Potassium L-homoserine, pH 9.0, was used in the homoserine tests, and 100 μmoles of potassium DL-homoserine were added in the DPN and TPN tests. The reaction was started, after adding the enzyme, by the addition of homoserine. Optical density readings were recorded every 30 seconds for several minutes.

A preparation of DL-glutamic γ-semialdehyde (9) was active in the test system at about 1 per cent of the ASA rate and showed the same DPNH-TPNH ratio found with ASA. The equivalent of about 3 μmoles per ml. of this substance was used.

p-Chloromercuribenzoate—Homoserine dehydrogenase activity was not affected by incubation with saturated *p*-chloromercuribenzoate at room temperature for 1 hour prior to the addition of either pyridine nucleotide

or any other substrate. The experiment was performed in 0.1 M potassium phosphate buffer, pH 6.7.

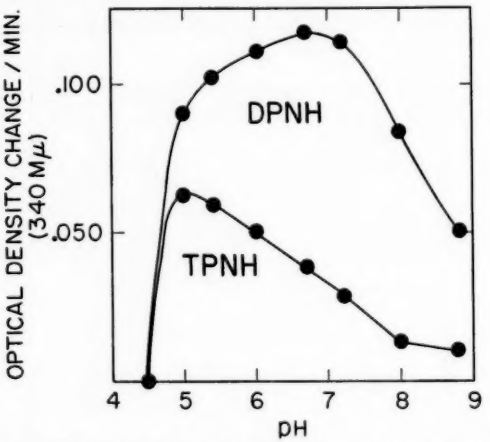


FIG. 4. Relation of reaction rate to pH. The enzyme test described in the text was used. Final buffer concentrations were all 0.1 M. Buffers used were K phthalate, K acetate, K succinate, K phosphate, imidazole chloride, Tris chloride, and diethanolamine chloride. The final pH was determined with a Beckman model G pH meter.

TABLE II

Heat Stability of Homoserine Dehydrogenase in Presence and Absence of Phosphate

Two small portions of the extract, one containing 0.15 M potassium phosphate, were held in a 60° water bath for 10 minutes, then cooled in an ice bath and centrifuged. The pH of both was 6.5.

	Enzyme, units per ml.		Enzyme recovery, per cent		Enzyme activity, ratio DPNH-TPNH
	DPNH	TPNH	DPNH	TPNH	
Extract	256	104			2.5
Supernatant, 60°	2.1	0.68	0.82	0.65	3.1
" with P, 60°	75	26	29	25	2.9

Equilibrium—The reversibility of the reaction is illustrated in Fig. 5. Addition of L-homoserine to the system with DPN or TPN causes reduction of the pyridine nucleotide until equilibrium is reached. Addition of L-ASA results in a lowering of the DPNH or TPNH concentration to a

second equilibrium level. The equilibrium constants

$$K \text{ (DPN or TPN)} = \frac{\text{homoserine} \times \text{DPN or TPN}}{\text{ASA} \times \text{DPNH or TPNH} \times \text{H}^+}$$

have been calculated from data obtained in the experiment shown in Fig.

TABLE III

Reduction Rate of DPN and TPN Separately and Combined

The experiment was performed as described for the DPN and TPN tests shown in Fig. 3.

Nucleotide added, μmole per ml.	Nucleotide reduction rate, μmole per min.
DPN (0.13)	0.020
TPN (1.00)	0.017
DPN + TPN (0.13 + 1.00)	0.021

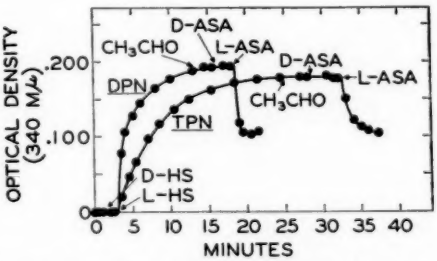


FIG. 5. Specificity and equilibrium of homoserine dehydrogenase. The reaction occurred at room temperature. The spectrophotometer cell contained initially 60 μmoles of Tris chloride buffer, 6.5 DPNH units of homoserine dehydrogenase, and 0.158 μmole of DPN or 0.214 μmole of TPN in 0.8 ml. At the points indicated, 10 μmoles of D- and L-homoserine were added, each in 0.1 ml. 0.01 ml. of 0.01 M acetaldehyde and 0.05 ml. each of 0.001 M D- and L-ASA were added as shown. All of the values plotted, and those shown in Table III, are corrected for dilution to correspond to a volume of 1.0 ml. The pH at the end of each test was found to be 7.9.

5. The second equilibrium point, reached after ASA addition, was used for the calculation. Final concentrations were calculated on the assumption that the amount of DPNH or TPNH formed was equivalent to ASA formation and to DPN or TPN and homoserine utilization. The K values for DPN and TPN, respectively, were found to be 0.9×10^{11} and 1.3×10^{11} (Table IV). A value for the analogous DPN-specific alcohol dehydrogenase reaction, calculated from the data of Racker (10), is 0.8×10^{11}; from the data of Negelein and Wulff (11) it was calculated to be 1.0×10^{11}.

ASA Determination—Fig. 5 shows the rapid utilization of DPNH which occurs when a small amount of L-ASA is added to the enzyme system. In this experiment the presence of initially added homoserine caused an equilibrium state to be reached before complete reduction of the L-ASA. When L-ASA is added to a system which contains DPNH and no homoserine, the disappearance of DPNH appears to be equivalent to the amount of L-ASA added. Thus, within the limits of experimental error, L-ASA was shown to be quantitatively formed by ozonolysis of L-allylglycine or by the enzymatic reduction of β-aspartyl phosphate (5).

Identification of Homoserine—By using the conditions of the enzyme test described, 1 μmole of DPNH was completely utilized in the presence of 1 μmole of ASA. The solution was then treated with 4 volumes of ethanol

TABLE IV

Equilibrium Data

The experiment is described in Fig. 5. All values, including H^+, are micromoles or microequivalents per ml.

	ASA	DPNH	H^+	Homoserine	DPN	K
Added	0.050			10.00	0.158	
At equilibrium	0.068	0.18	1.26×10^{-5}	9.98	0.140	0.9×10^{11}
		TPNH			TPN	
Added	0.050			10.00	0.214	
At equilibrium	0.068	0.018	1.26×10^{-5}	9.98	0.196	1.3×10^{11}

centrifuged to remove the precipitate, and evaporated to a small volume. Homoserine was identified in this solution by paper chromatography in solvents previously described (4). No homoserine was found in control experiments without ASA or DPNH. In a parallel experiment with TPNH, homoserine was also identified as the product.

Homoserine is easily distinguished from threonine by using 85 per cent *n*-propanol plus diethylamine (12) to develop paper chromatograms of these substances. R_F values are, respectively, 0.43 and 0.52.

DISCUSSION

The available evidence suggests that a single enzyme is involved in the reactions with either of the two pyridine nucleotides, TPN and DPN. This evidence, which is not conclusive, may be summarized as follows. The ratio of the two activities did not change significantly during a 100-fold purification. It also did not change when over 99 per cent of the enzyme was destroyed by heat. Both activities were partially and equally

protected from heat denaturation by the presence of inorganic phosphate. When both nucleotides were added to the reaction mixture, there was no summation of the rates of the individual reactions; this was true in the reverse as well as in the forward reaction. The maximal rates for the forward or reverse reactions, calculated according to Lineweaver and Burk (8), are approximately equal for the two nucleotides.

The two following observations are not readily explained by the one enzyme hypothesis, though they are not necessarily incompatible with it. A 7-fold difference in the K_m values for homoserine was found when these values were determined with TPN and DPN. A different pH optimum for the forward reaction was found by using TPNH from that observed with DPNH.

The insensitivity of homoserine dehydrogenase to *p*-chloromercuribenzoate is unusual for a pyridine nucleotide-linked enzyme (13).

Materials and Methods

Glutamic γ-semialdehyde was prepared by autoclaving 37 mg. of γ,γ-dicarbethoxy-γ-acetamidobutyraldehyde (9) for 20 minutes in 0.7 ml. of 6 N HCl and neutralizing with an excess of potassium acetate. The unhydrolyzed precursor was obtained from Dr. Alton Meister. 3-Aminopropanol was obtained from the Aldrich Chemical Company (Milwaukee). Potassium γ-hydroxybutyrate was obtained by mixing KOH with γ-butyrolactone. The latter was a commercial preparation supplied by Dr. Herbert Tabor. The D and L isomers of homoserine were generously supplied by Dr. J. P. Greenstein. TPN ("80"), DPNH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were products of the Sigma Chemical Company. Bovine serum albumin was obtained from the Armour Laboratories.

TPNH was generally prepared according to Kaplan *et al.* (14). However, this preparation contains an inhibitor which competes with ASA when the concentration of the latter is low. For certain experiments, therefore, TPNH was formed enzymatically with glucose-6 phosphate dehydrogenase (15) in Tris buffer, pH 8.0. 0.2 μmole of TPN and of glucose-6 phosphate each was added per ml. When reduction was over 90 per cent complete, the resulting solution was used directly in experiments.

Protein was determined by the optical method of Warburg and Christian (16). DPNH and TPNH were determined by their absorption of light at 340 mμ. An extinction coefficient of 6.22×10^6 sq. cm. per mole (17) was assumed for each substance. The measurements were made in a volume of 1.0 ml. in a quartz cell[2] having inside dimensions of $10 \times 5 \times 38$ mm.

[2] These cells may be obtained from the Pyrocell Manufacturing Company, 207 East 48th Street, New York 28.

The light path was 10 mm. DPN was determined enzymatically by Racker's method (10) and TPN by a similar method with glucose-6-phosphate dehydrogenase (15).

SUMMARY

1. Homoserine dehydrogenase catalyzes the reversible reduction of aspartic β-semialdehyde (ASA) to homoserine by DPNH or TPNH.

2. This enzyme has been purified over 100-fold from a yeast extract without a significant change in the ratio of activities with DPNH and TPNH. The two activities are equally labile to heat and equally protected from heat destruction by 0.15 M potassium phosphate.

3. The enzyme is specific for the L isomers of ASA and homoserine. DPN is not reduced, in the presence of the enzyme, by 3-aminopropanol or γ-hydroxybutyrate. It appears to have a small activity with glutamic γ-semialdehyde.

4. K_m values for all substrates have been determined. Of particular interest is a 7-fold difference in the K_m values for homoserine when tested with DPN or TPN.

5. Equilibrium constants for the reaction, with DPN and TPN, have been found to be very similar to constants reported for the analogous alcohol dehydrogenase reaction.

We wish to thank Dr. H. A. Sober and Dr. E. A. Peterson for instruction on the preparation and use of the calcium phosphate column, and Dr. A. H. Mehler for valuable suggestions concerning some of the experiments.

BIBLIOGRAPHY

1. Black, S., and Wright, N. G., *Federation Proc.*, **13,** 184 (1954).
2. Black, S., and Wright, N. G., *J. Am. Chem. Soc.*, **75,** 5766 (1953).
3. Black, S., and Gray, N. M., *J. Am. Chem. Soc.*, **75,** 2271 (1953).
4. Black, S., and Wright, N. G., *J. Biol. Chem.*, **213,** 27 (1955).
5. Black, S., and Wright, N. G., *J. Biol. Chem.*, **213,** 39 (1955).
6. Sober, H. A., and Peterson, E. A., *J. Am. Chem. Soc.*, **76,** 1711 (1954).
7. Price, V. E., and Greenfield, R. E., *J. Biol. Chem.*, **209,** 363 (1954).
8. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
9. Vogel, H. J., and Davis, B. D., *J. Am. Chem. Soc.*, **74,** 109 (1952).
10. Racker, E., *J. Biol. Chem.*, **184,** 313 (1950).
11. Negelein, E., and Wulff, H., *Biochem. Z.*, **293,** 351 (1937).
12. Redfield, R. R., *Biochim. et biophys. acta*, **10,** 344 (1953).
13. Neilands, J. B., *J. Biol. Chem.*, **208,** 225 (1954).
14. Kaplan, N. O., Colowick, S. P., and Neufeld, E. F., *J. Biol. Chem.*, **195,** 107 (1952).
15. Warburg, O., and Christian, W., *Biochem. Z.*, **292,** 287 (1937).
16. Warburg, O., and Christian, W., *Biochem. Z.*, **310,** 384 (1941).
17. Horecker, B. L., and Kornberg, A., *J. Biol. Chem.*, **175,** 385 (1948).

STUDIES ON THE ORIGIN OF ERGOTHIONEINE IN ANIMALS*

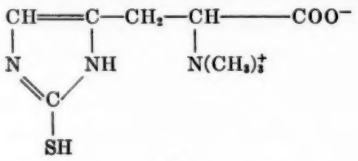
By DONALD B. MELVILLE, WILLIAM H. HORNER, CHARLES C. OTKEN, and MARTHA L. LUDWIG†

(From the Department of Biochemistry, Cornell University Medical College, New York, New York)

(Received for publication, August 5, 1954)

The wide-spread occurrence of ergothioneine in animals (1–3) suggests that the compound is either synthesized by several animal species or is present in common foodstuffs. However, data in the literature on this point are equivocal and contradictory. Eagles and Vars (4) suggested that the corn protein, zein, contains 2-thiolhistidine which serves as a dietary precursor of ergothioneine. Repeated attempts by other workers to identify thiolhistidine as a constituent of corn have failed (5–7). The conclusion of Potter and Franke (8) that corn-containing diets lead to increased blood ergothioneine levels in rats, and the similar conclusion of Eagles and Vars with regard to pigs, could not be confirmed by Hunter (6).

Consideration of the structure of ergothioneine (I) suggests as likely



(I)

precursors the amino acids histidine and 2-thiolhistidine, the methyl group of methionine, and the sulfur atom of methionine or cystine. In this paper we report studies undertaken to determine whether ergothioneine is synthesized in animal tissues from such precursors, or whether it is of solely dietary origin. Our results strongly indicate that ergothioneine is not synthesized by the rat, chicken, or guinea pig. While this work was in progress, Heath and coworkers (9) published data from which they concluded that the boar is capable of synthesizing ergothioneine.

EXPERIMENTAL

Materials—L-2-Thiolhistidine was prepared from L-histidine by modifications of the method of Ashley and Harington (5). L-Histidine labeled

* This work was aided by grants from the Nutrition Foundation, Inc., and Eli Lilly and Company.

† Fellow of the Helen Hay Whitney Foundation.

with C^{14} in the imidazole ring was prepared from $NaC^{14}N$ by a method similar to that used by Borsook and coworkers (10) and was crystallized as the dihydrochloride from acetic acid ($[\alpha]_D^{20}$ +6.68°, c = 14 in water; an authentic sample of L-histidine dihydrochloride gave +6.65° under similar conditions). L-Methionine labeled in the methyl group with C^{14} was prepared by the method of Melville, Rachele, and Keller (11). L-Methionine labeled with S^{35} was purchased from the Abbott Laboratories, North Chicago, Illinois.

Methods—Blood and liver were examined for ergothioneine by the chromatographic method previously described (3, 7). Briefly, this consists of treatment of the lyzed cells or homogenized tissue with glutathione and dithionite, precipitation of the proteins with trichloroacetic acid, treatment of the filtrate with IRA-410 acetate, and chromatography of the resulting material on alumina. The developing solvent was either 75 per cent aqueous ethanol, 80 per cent aqueous ethanol, or 75 per cent aqueous ethanol containing 1 per cent formic acid (3). The effluent from the columns was collected in 1 ml. fractions, and alternate tubes were analyzed for ergothioneine by means of the color reaction with diazotized sulfanilic acid (12). The remaining fractions were tested for radioactivity by evaporation to dryness in a thin film on 25 mm. watch glasses and examination in a windowless flow counter. In most cases the residues weighed 0.2 mg. or less and were spread over an area of approximately 1 cm.^{2}

We have shown previously that the ergothioneine of rat tissues decreases to undetectable levels when the animals are fed purified diets containing casein as the source of protein (3). While this fact in itself suggests that ergothioneine cannot be synthesized by the rat, the possibility remains that purified diets lack a factor which is present in ordinary diets and which is essential for the synthesis of ergothioneine. To determine whether this factor might be L-2-thiolhistidine, two adult Sherman strain rats were placed on the purified diet and each was injected daily with 5 mg. of thiolhistidine for a period of 27 days. At the end of this time neither animal showed any detectable blood ergothioneine by the direct method of determination (12).

Administration of Histidine-2-C^{14} to Rat—A neutralized solution of 181 mg. of L-histidine-2-C^{14} dihydrochloride, possessing approximately 2×10^7 c.p.m. as determined by direct count on a 7 γ aliquot, was injected subcutaneously in equal daily doses for a period of 20 days into a 260 gm. Sherman strain male rat. The animal was maintained on a commercial stock diet (Rockland rat diet) which we have found to be adequate in maintaining blood ergothioneine levels. At the end of the injection period 8 ml. of blood were subjected to the chromatographic analysis procedure. The results of the ergothioneine and radioactivity determinations are shown in Fig. 1.

Administration of C^{14}-Labeled Methionine to Rat—An aqueous solution of 100 mg. of L-methionine labeled in the methyl group with C^{14} and showing approximately 2.2×10^7 c.p.m. was injected subcutaneously into a 115 gm. Sherman strain male rat, maintained on the commercial stock diet, in equal daily doses for 20 days. At the end of this time blood from the animal was analyzed chromatographically. It was found that a radioactive peak partly overlapped the ergothioneine peak area. The liver from the same animal was therefore analyzed, but again a radioactive peak overlapped the ascending portion of the ergothioneine curve. The radioactive material was separated effectively from the ergothioneine by com-

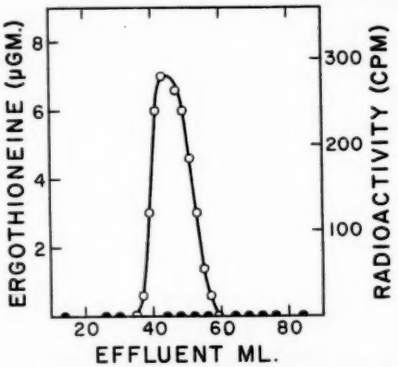


FIG. 1. Distribution of ergothioneine (○) and radioactivity (●) in alumina chromatogram of red cells from rat which had received C^{14}-labeled histidine.

bining and rechromatographing the alternate fractions in the ergothioneine area which had been used for radioactivity determinations (Fig. 2).

A small amount of residual radioactivity was present in the fractions containing the ergothioneine. To determine whether this activity was present in the methyl groups of the ergothioneine, use was made of the fact that the methyl groups can be removed readily as trimethylamine by treatment of ergothioneine with strong alkali. The residue from Fraction 66, which contained more than 8 γ of ergothioneine and showed an activity of 10 c.p.m., was heated in one arm of a small, sealed U-tube with a few drops of concentrated KOH solution at 60° for 1 hour. The second arm of the apparatus contained a few drops of 6 N HCl to trap the trimethylamine. After a diffusion period of 3 hours the HCl solution was evaporated to dryness on a watch glass and the residue was examined in the flow counter. No detectable radioactivity was present.

Administration of C^{14}-Labeled Methionine to Chicken—A 70 gm., week-old "broad breast" chicken was maintained on a commercial ration (Ralston

Purina Company, Growena) and injected subcutaneously with the L-methionine labeled in the methyl group with C^{14}. The dosage and injection schedule were the same as those employed with the rat. At the end of the injection period the blood was analyzed. In this case, several of the fractions in the ergothioneine range showed no detectable radioactivity above the background count (Fig. 2).

Administration of S^{35}-Labeled Methionine to Rat—A 322 gm. Wistar strain male rat was maintained on the stock diet and was injected subcutaneously with a total of 45.5 mg. of S^{35}-labeled L-methionine over a 20 day period in equal daily doses. The total radioactivity administered was

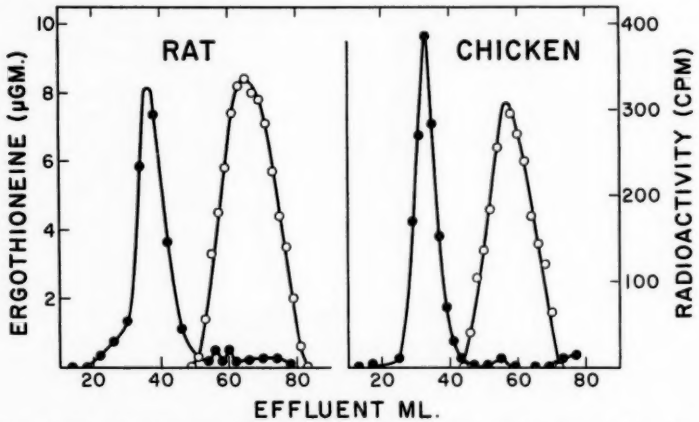


FIG. 2. Distribution of ergothioneine (○) and radioactivity (●) in alumina chromatograms of rat liver and chicken red cells from animals which had received methyl-labeled C^{14}-methionine.

approximately 2×10^8 c.p.m. as determined by direct count on a small aliquot. The results of the analysis of blood obtained from the animal at the end of the injection period are shown in Fig. 3.

Administration of S^{35}-Labeled Methionine to Guinea Pig—A 245 gm. guinea pig was maintained on commercial rabbit pellets (Rockland) supplemented with cabbage and was injected with the S^{35}-methionine at the same dosage and radioactivity levels used with the rat. The analytical results are shown in Fig. 3.

In addition to the foregoing experiments, analyses were carried out on blood samples which became available from work not connected with the ergothioneine problem, but in which isotope-labeled compounds had been administered.

Analysis of Blood from Human Given S^{35}-Cystine—A sample of red cells

was obtained from a 46 year-old male patient with lymphocytic lymphoma, who had been given 0.4 mc. (18.2 mg.) of S^{35}-labeled L-cystine by mouth. The blood sample had been collected 21 hours after administration of the cystine. Chromatographic analysis yielded a column effluent containing a well-defined ergothioneine peak with a maximum of 22 γ of ergothioneine in one of the fractions. This fraction showed no detectable radioactivity in the flow counter.

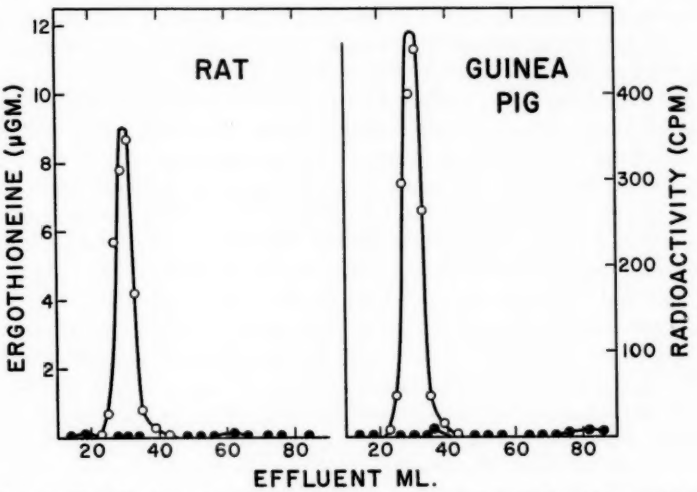


FIG. 3. Distribution of ergothioneine (○) and radioactivity (●) in alumina chromatograms of red cells from rat and guinea pig which had received S^{35}-labeled methionine.

Analysis of Blood from Rat Given C^{14}-Glycine—Blood was obtained from a 370 gm. Sherman strain male rat, maintained on a commercial stock diet, which had undergone partial hepatectomy 31 days earlier and which had been injected intraperitoneally for the first 2 postoperative days with a total of approximately 0.01 mc. (0.54 mg.) of glycine-2-C^{14}. After analysis, the effluent fraction containing the largest amount of ergothioneine (11 γ) was examined, but no radioactivity could be detected.

Analysis of Blood from Rat Given C^{14}-Formaldehyde—Blood was obtained from a 330 gm. Sherman strain male rat, maintained on a commercial stock diet, which had been injected subcutaneously three times daily for 10 days with a total of 5.7 mmoles of formaldehyde-C^{14},D_2 (a mixture of $C^{14}H_2O$ and CD_2O) having a total activity of approximately 5.8×10^6 c.p.m. The animal was killed 21 hours after the last injection and the

blood was analyzed. The effluent fraction containing the largest amount of ergothioneine (17 γ) was found to contain no detectable radioactivity.

DISCUSSION

Our finding of the inability of injected 2-thiolhistidine to support blood ergothioneine levels is in conformity with the work of Heath (13).

The experiments described have all given uniformly negative results in our attempts to demonstrate the synthesis of ergothioneine in the rat, the chicken, the guinea pig, and the human. In the cases of the rat, chicken, and guinea pig, the radioactive compounds were injected over long periods of time to minimize the possibility of inability to detect synthesis because of the slow rate of incorporation of ergothioneine into the red cell (14). It is estimated that, in the experiment with C^{14}-histidine, significant levels of radioactivity would have appeared in the ergothioneine if as little as 2 per cent of the blood ergothioneine had arisen from the radioactive histidine; in the S^{35}-methionine experiment labeling would have been readily detected if as little as 1 part in 1000 of the sulfur of the blood ergothioneine had been derived from the administered methionine.

The negative results obtained with the rat and the chicken after the administration of methyl-labeled methionine (Fig. 2) show that the methyl groups of ergothioneine are not derived from transmethylation reactions with compounds containing labile methyl groups. Whether ergothioneine can act as a methyl group donor has not been determined. The strongly radioactive peak evident in Fig. 2 in the chromatograms for both the rat and the chicken is due to a ninhydrin-negative substance which is undoubtedly choline, inasmuch as C^{14}-labeled choline was found to behave identically as the unknown substance on the chromatograph columns. These results are therefore an excellent demonstration of the difference in availability of the methyl group of methionine for transmethylation to choline and to ergothioneine.

The negative results obtained with ring-labeled histidine and methyl-labeled methionine did not exclude the possibility that hercynine, the naturally occurring betaine of histidine, might serve as a dietary precursor of ergothioneine. However, the negative findings with S^{35}-methionine in the rat and the guinea pig (Fig. 3) rule out such a possibility. The results obtained with C^{14}-glycine and C^{14}-formaldehyde, as well as with methyl-labeled methionine, suggest that single carbon compounds are not involved. In the experiment with S^{35}-cystine in the human, the negative results can be regarded as only suggestive of the non-synthesis of ergothioneine, because of the short term nature of the experiment and the high dilution factors which are involved.

It would seem reasonable to conclude that ergothioneine is not synthe-

sized in the tissues of the rat, chicken, or guinea pig. The possibility that the compound might arise from synthesis by microorganisms of the intestinal tract seems doubtful, since we have found that blood ergothioneine levels in the rat are not significantly altered by the feeding of sulfasuxidine and have shown, by studies on germ-free chickens, that the intestinal flora do not contribute significant amounts of ergothioneine to the blood in this species (7).

It appears most likely, therefore, that ergothioneine is of dietary origin in these animals. There is evidence in the literature to indicate that cereal grains are effective to varying degrees as dietary sources of ergothioneine (8); the recent work of Baldridge and Lewis (15) and Baldridge (16) implicates oats as being a good source. The failure so far to identify ergothioneine as a constituent of plant materials may be due to low concentrations of the substance or possibly to its occurrence in a bound form.

The work presented by Heath and coworkers (9) to show that S^{35}-methionine gives rise to radioactive ergothioneine in the pig is unexpected in view of our present results and indicates the need for further studies of species differences in the ability to synthesize ergothioneine.

The authors express their appreciation to Dr. Aaron Bendich and Dr. Jacques R. Fresco for supplying the blood from glycine-treated animals, to Dr. Bertram A. Lowy for blood from formaldehyde-treated animals, and to Dr. Laurance Goodwin for blood from the cystine-treated human subject.

SUMMARY

By the administration of isotope-labeled compounds considered to be likely precursors, studies on the possible synthesis of ergothioneine in animals have been carried out. No evidence for the formation of isotope-labeled ergothioneine has been obtained after the administration of C^{14}-labeled histidine to the rat, methyl-labeled C^{14}-methionine to the rat and the chicken, S^{35}-labeled methionine to the rat and the guinea pig, C^{14}-labeled glycine or C^{14}-labeled formaldehyde to the rat, and S^{35}-labeled cystine to the human.

BIBLIOGRAPHY

1. Hunter, G., *Biochem. J.*, **22,** 4 (1928).
2. Mann, T., and Leone, E., *Biochem. J.*, **53,** 140 (1953).
3. Melville, D. B., Horner, W. H., and Lubschez, R., *J. Biol. Chem.*, **206,** 221 (1954).
4. Eagles, B. A., and Vars, H. M., *J. Biol. Chem.*, **80,** 615 (1928).
5. Ashley, J. N., and Harington, C. R., *J. Chem. Soc.*, 2586 (1930).
6. Hunter, G., *Biochem. J.*, **48,** 265 (1951).
7. Melville, D. B., and Horner, W. H., *J. Biol. Chem.*, **202,** 187 (1953).

8. Potter, V. R., and Franke, K. W., *J. Nutr.*, **9,** 1 (1935).
9. Heath, H., Rimington, C., Glover, T., Mann, T., and Leone, E., *Biochem. J.*, **54,** 606 (1953).
10. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **187,** 839 (1950).
11. Melville, D. B., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169,** 419 (1947).
12. Melville, D. B., and Lubschez, R., *J. Biol. Chem.*, **200,** 275 (1953).
13. Heath, H., *Biochem. J.*, **54,** 689 (1953).
14. Heath, H., Rimington, C., Searle, C. E., and Lawson, A., *Biochem. J.*, **50,** 530 (1952).
15. Baldridge, R. C., and Lewis, H. B., *J. Biol. Chem.*, **202,** 169 (1953).
16. Baldridge, R. C., *Federation Proc.*, **13,** 178 (1954).

THE DETERMINATION OF TOTAL LIPIDES IN BLOOD SERUM*

By WARREN M. SPERRY and FLORENCE C. BRAND

(From the Departments of Biochemistry, New York State Psychiatric Institute, and the College of Physicians and Surgeons, Columbia University, New York, New York)

(Received for publication, July 2, 1954)

There is no well established method for the direct determination of unmodified total lipides in blood serum or plasma. A few gravimetric methods have been proposed (1–5), but in none is an adequate procedure of purification included, and each is open to possible criticism on other grounds. None has been tested by extensive application.

The most widely used methods for the determination of total lipides in the blood serum have developed from the procedure of Bang (6), who oxidized the lipides with acid chromate and measured the chromate consumed by iodometry. Under Bang's conditions, the oxidation was incomplete and an empirical factor had to be applied in calculating the results. Bloor (7) made the outstanding contribution of finding conditions under which the oxidation is nearly quantitative. In his popular procedure the lipides are saponified, and the unsaponifiable material and fatty acids are oxidized. The so called "total lipides" are calculated from the cholesterol concentration and theoretical factors for the oxidation of the cholesterol-fatty acid mixture. Recently, Bragdon (8), employing a modification of the colorimetric procedure (9) for measuring the oxidation, applied the method to the unsaponified lipides of the serum. Determinations of cholesterol and lipide phosphorus are also necessary for calculation of the results on the basis of oxidation factors. The estimation of total lipides in this and other modifications of the oxidation method is based on the assumption of molecular weights of the complex mixture of lipides which are present in the serum.

It is evident that there is a need for a method which permits the direct determination of the unmodified lipides of the blood serum. In such a method (*a*) extraction of lipides should be complete, (*b*) they should be completely freed of non-lipide contaminants, (*c*) oxidative degradation should be avoided, (*d*) a small quantity of serum should be required, (*e*) the analytical procedure should be reasonably simple, and (*f*) the results should be accurate. A procedure which satisfies these criteria is described

* This investigation was supported in part by a grant from the National Multiple Sclerosis Society.

in this communication. It is based, to a large extent, on a method (10) recently described for the determination of total lipides in brain; the techniques of purification, transfer, filtration, evaporation to dryness, and weighing are the same unless otherwise noted.

Procedure

1. Extraction—Into 8.3 ml. of pure methanol in a 25 ml. volumetric flask is pipetted 1 ml. of blood serum or plasma. The tip of the pipette is held just above the junction of the neck and the bulb, and the flask is swirled during the addition. An approximately equal volume of chloroform is added, and the solvent is brought just to a boil on the steam bath. After cooling the flask to room temperature, chloroform is added to the mark, and the contents are thoroughly mixed and filtered.

2. Purification of Lipide Extract—Two procedures are described. In Procedure 1, 20 ml. of extract are pipetted into a vial, approximately 2 cm. in diameter and 8 cm. high, water is added slowly (*cf.* (10)) until the vial is full and the vial is lowered with forceps into a rack at the bottom of a 1 liter beaker which is almost full of water. Diffusion is allowed to proceed for at least 18 hours. The "fluff" which usually collects in small amount at the interface is more easily loosened into suspension in the upper phase by agitation than is the case with brain lipide extracts. To avoid this danger of loss, the 1 liter beaker is carefully placed on a Cenco-Lerner Lab Jack, water is removed by suction until the level is below the rim of the vial, the Van Slyke-Rieben pipette (11) is centered over the vial, and the beaker is raised by the Lab Jack until most of the upper phase has been removed. Good illumination should be used, and a magnifier is desirable. It is necessary to watch carefully for any suspension which may occur as the meniscus reaches "fluff" adhering to the wall of the vial. If any tendency toward suspension is seen, the pipette is removed at once by lowering the beaker.

Procedure 2 is based on the revised method of Folch *et al.* (12), and further information concerning it which was kindly supplied by Dr. Folch in a personal communication. 20 ml. of the filtrate are pipetted into a 25 ml. glass-stoppered cylinder, previously tested for tightness of the stopper, 4 ml. of water are added, the stopper is moistened with water and tightly inserted, and the contents are shaken vigorously for 1 minute. The cylinder is allowed to stand overnight or until the two phases have completely separated. The upper phase should be clear; the lower phase usually also becomes clear, but it may remain slightly cloudy, some droplets of upper phase adhering to the wall of the cylinder. The upper phase is removed as completely as possible with the Van Slyke-Rieben pipette. This operation is carried out in our laboratory with the aid of a Lab Jack as described in the preceding paragraph, but could doubtless be done by carefully raising the cylinder by hand since no "fluff" is present.

Because the volume of the upper phase containing the non-lipide impurities is small, in contrast to the conditions of Procedure 1, the residue must be removed by washing. A solution is prepared for this purpose as follows: 96.3 ml. of chloroform-methanol (2:1) and 23.7 ml. of a solution containing 20 mg. of $CaCl_2$ per 100 ml. of water are thoroughly equilibrated in a separatory funnel and allowed to stand until the phases have separated and the upper phase is clear. The lower phase is discarded. The remaining solution has approximately the same solvent concentration as the upper phase obtained in the purification of the lipide extracts. 3 ml. of this solution are added to the cylinder; the pipette tip is placed against the neck, and the cylinder is turned as the solution is slowly added so the entire wall is washed. Care is taken to avoid mixing with the lower phase (*cf.* (12)). The cylinder is briskly rotated back and forth in a vertical position to mix the residue of the original upper phase with the wash solution, which is removed as before. The washing procedure is repeated twice more.

3. Evaporation to Dryness, Filtration, and Weighing—If Procedure 1 is used, 6 ml. of methanol are added to the vial, and the mixture is stirred. A clear solution usually results, but a little more methanol may be required if an exceptionally large amount of water was left at step (2). The solution is transferred to a 50 ml. flask and evaporated to dryness *in vacuo* in an atmosphere of nitrogen.

If Procedure 2 is used, 2 ml. of methanol are added to the cylinder, the contents are mixed, and the clear solution is transferred to a 50 ml. Erlenmeyer flask, chloroform-methanol (2:1) being used for washing. The solution is evaporated to dryness at 35–40° in a stream of nitrogen.

The lipides obtained by either purification procedure are dissolved in chloroform-methanol, filtered into 5 ml. volumetric flasks, dried, and weighed, as previously described (10), except that after evacuation of the desiccator nitrogen is admitted, and the desiccator is evacuated again. This is repeated twice more. The weight of lipides multiplied by 125 gives the concentration in mg. per 100 ml.

EXPERIMENTAL

The method was tested by determining the recoveries of phosphatides and total and free cholesterol and by comparison with a somewhat different procedure. From each sample of pooled serum or plasma, extracts were prepared in ethanol-ether (3:1), ethanol-acetone (1:1), and chloroform-methanol (2:1) as described above. Phosphorus was determined in the ethanol-ether extract by the method of Sperry (13), and the cholesterol fractions were determined in the ethanol-acetone extract by the method of Sperry and Webb (14). In some experiments, the chloroform-methanol extract was made from 2 ml. of serum or plasma in a 50 ml. volumetric flask to provide sufficient volume for phosphorus and cholesterol determina-

tions in the same solution in which the total lipides were measured. At the end of the procedure, the weighed lipides were dissolved in chloroform-methanol (2:1) and transferred quantitatively to a 25 ml. volumetric flask. After dilution to volume, suitable aliquots were taken for phosphorus and cholesterol analyses. When cholesterol was determined in chloroform-methanol solutions, aliquots were pipetted into 12 ml. centrifuge tubes, the solvent was evaporated at 35–40° by a stream of nitrogen, and either 1 ml. or 2 ml. of ethanol-acetone (1:1) were added for total or free cholesterol determinations, respectively.

TABLE I

Recovery of Phosphatides

Serum or plasma No.	Phosphorus			Recovery based on	
	In ethanol-ether extracts	In chloroform-methanol extracts	In weighed lipides	Ethanol-ether extracts	Chloroform-methanol extracts
	mg. per 100 ml.	*mg. per 100 ml.*	*mg. per 100 ml.*	*per cent*	*per cent*
4	8.24		8.21	99.6	
5	10.68		11.04	103.4	
6	8.87	9.03	9.21	103.8	102.0
7	9.10	9.53	9.35	102.7	98.1
8	10.51	10.49	10.17	96.8	96.9
9	9.67	9.53	9.33	96.5	97.9
10	7.67	7.32	7.34	95.7	100.3
11	9.15		9.35	102.2	
12	7.80		7.63	97.8	
13	6.78		6.53	96.3	
14	12.48		12.08	96.8	
15	8.83		8.62	97.6	
Average recovery				99.1	99.0

Recovery of Phosphatides—The results for phosphorus (Table I) show that the average recovery was almost quantitative. They also provide further support for the conclusion that negligible amounts of non-lipide, phosphorus-containing compounds are present in crude extracts of blood serum or plasma (*cf.* (15)), since such compounds would almost certainly be removed by the purification procedures.

Recovery of Cholesterol—Although in some experiments the recovery of cholesterol was quantitative, in the majority the results were unsatisfactory. The difficulty was traced in part to degradation of cholesterol, which may be greater than is generally realized when dry lipides are exposed to air. This is illustrated by the following experiment. 5 ml. of serum were extracted in chloroform-methanol (2:1) in a 100 ml. volumetric flask by the

procedure described above. For control determinations, 1 ml. of the same serum was extracted in ethanol-acetone (1:1) in a 25 ml. volumetric flask. Four 20 ml. aliquots (Samples A, B, C, and D) of the chloroform-methanol extract were purified by Procedure 1, taken to dryness *in vacuo*, and dissolved in chloroform-methanol. Sample A was filtered into a 25 ml. volumetric flask. Solvent was added almost to the mark, and the solution was stored. Samples B, C, and D were carried through the remainder of the procedure as described above. After being weighed, Sample B was dissolved at once in chloroform-methanol, transferred to a 25 ml. volumetric flask, and stored under the same conditions as Sample A. Sample C was returned to the Dri-Jar desiccator[1] and left there in dim light for 42 hours. At the end of this time, the weight had not changed (6.48 *versus* 6.46 mg.).

TABLE II

Recovery of Cholesterol

Sample*	Cholesterol		Recovery	
	Total	Free	Total cholesterol	Free cholesterol
	mg. per 100 ml.	*mg. per 100 ml.*	*per cent*	*per cent*
Control	166.3	45.9		
A	166.5	46.8	100.1	102.0
B	165.4	44.9	99.5	97.8
C	145.9	34.5	87.7	75.2
D	128.4	29.3	77.2	63.8

* The treatment of these samples is described in the text.

Sample D was left open in the laboratory for 42 hours. Samples C and D were dissolved in chloroform-methanol and transferred to 25 ml. volumetric flasks. Duplicate aliquots of Samples A, B, C, and D and of the control extract in ethanol-acetone were pipetted at the same time for cholesterol analysis. The results (Table II) show that there was no loss of cholesterol during the purification (Sample A) or in the entire procedure (Sample B), but when the dried lipides were exposed to air recoveries were poor (Samples C and D).

Comparison with Different Extraction Procedure—25 or 50 ml. extracts of pooled serum or plasma were prepared in ethanol-ether (3:1) and in chloroform-methanol (2:1). An aliquot of 20 or 25 ml. of the filtered ethanol-ether extract was evaporated to dryness *in vacuo* in an atmosphere of nitrogen. The residue from the 25 ml. aliquots was extracted with chloroform methanol (2:1) and filtered into a 25 ml. volumetric flask. A 20 ml.

[1] The Dri-Jar desiccators were obtained from the Bethlehem Apparatus Company, Hellertown, Pennsylvania.

portion was pipetted either into a vial for Procedure 1 or a 25 ml. cylinder for Procedure 2. When a 20 ml. aliquot was used, the extract of the dried residue was filtered directly into a vial or cylinder, and chloroform-methanol was added to a volume of approximately 20 ml. The subsequent procedure was the same as that described in the proposed method.

When Procedure 2 was used for purification of ethanol-ether extracts, the total lipide values agreed closely with those found by either method of purification in the proposed method (Table III). When Procedure 1 was used, the weights of lipides from ethanol-ether extracts were consistently

TABLE III

Comparison of Proposed Method with Different Extraction Procedure

Serum or plasma No.	Total lipides		Comparison $\frac{A}{B} \times 100$
	Proposed method* (*A*)	Extraction with ethanol-ether† (*B*)	
	mg. per 100 ml.	*mg. per 100 ml.*	
7	739	748	98.8
9	730	746	97.9
10	592	595	99.5
11	715	718	99.6
12	666	661	100.8
13	520	519	100.2
14	1043	1055	98.9
15	798	816	97.8
Average			99.2

* Average of values obtained with Procedures 1 and 2.

† Values obtained with Procedure 2.

higher. This unexplained result is probably related to the partial insolubility in chloroform-methanol of these lipides after weighing and to the presence of much more "fluff" in the purification of ethanol-ether than of chloroform-methanol extracts.

Determination of Urea in Total Lipides—Christensen (16) and Folch and Van Slyke (17) showed that blood serum or plasma lipides, purified by the conventional procedure of extracting dried ethanol-ether extracts with petroleum ether, still contained large amounts of non-lipide contaminants, of which urea was the principal constituent. The absence of significant amounts of urea in the lipides obtained in the proposed method was established. Solutions of lipides remaining after removal of samples for phosphorus and cholesterol determination were pooled and analyzed for urea by a modification of the method of Sobel *et al.* (18). The precision of the

method was such that well under 0.5 per cent of urea in the amount of lipides analyzed would have been detected. None was found in lipides purified either by Procedure 1 or 2. From this result, together with the findings of Christensen (16), Folch and Van Slyke (17), and Folch *et al.* (19), it is reasonable to assume that all water-soluble compounds were removed from the lipides by the purification procedures.

DISCUSSION

Serum is extracted in methanol, and chloroform is added later, because a much more finely divided precipitate is thus obtained. It is possible that extraction would be equally complete if made directly in chloroform-methanol (2:1).

The method is made possible by the availability of adequate procedures for purification of the lipides from non-lipide contaminants (*cf.* (16, 17)). The two procedures give the same result: in nine comparisons the average amount of lipides obtained by Procedure 2 was 100.2 per cent of the weight obtained when Procedure 1 was used. The choice between them must, therefore, be made on the basis of their advantages and disadvantages in terms of time and convenience. Procedure 1 requires more bench space to accommodate the large beakers, but considerably less working time, since no washing is required. With Procedure 2 the total time for an analysis could be considerably shortened if centrifuging instead of standing overnight were used to separate the phases, but the working time would be increased by this modification.

In developing the method a good deal of trouble was experienced with a partial insolubility of the weighed lipides in chloroform-methanol, in contrast to the experience with brain lipides, which have always been completely soluble at the end of an analysis (10). This difficulty was almost entirely eliminated by replacing the residual air in the desiccator by nitrogen, a technique which appears to be necessary also to prevent degradation of cholesterol. If the lipides are weighed within 1 hour after the flasks are placed in the Dri-Jar desiccator, and if they are dissolved in chloroform-methanol immediately after weighing, they may be used for analyses of their constituents with little danger of appreciable degradation.

The authors are indebted to Dr. Jordi Folch for suggestions concerning the application of the modified purification procedure (13) to blood serum, with particular reference to the preparation of the wash solution.

SUMMARY

A method is described for the direct, gravimetric determination of the unmodified total lipides of blood serum or plasma. Oxidative degradation

XUM

is avoided, and the lipides are freed of non-lipide contaminants. They may be used for the analysis of their constituents, and the method is, therefore, economical of material.

Evidence is presented for a high susceptibility of cholesterol to oxidative degradation.

BIBLIOGRAPHY

1. Wilson, W. R., and Hanner, J. P., *J. Biol. Chem.*, **106,** 323 (1934).
2. Kien, G., and Wetzler-Ligeti, C., *Wien klin. Wochschr.*, **48,** 871 (1935).
3. Street, H. R., *J. Biol. Chem.*, **116,** 25 (1936).
4. Mata, M., *Rev. farm. Cuba*, **26,** 29 (1948); *Chem. Abstr.*, **43,** 3871 (1949).
5. Cremer, H. D., *Klin. Wochschr.*, **27,** 755 (1949).
6. Bang, I., *Biochem. Z.*, **91,** 86, 235 (1918).
7. Bloor, W. R., *J. Biol. Chem.*, **77,** 53 (1928).
8. Bragdon, J. H., *J. Biol. Chem.*, **190,** 513 (1951).
9. Bloor, W. R., *J. Biol. Chem.*, **170,** 671 (1947).
10. Sperry, W. M., *J. Biol. Chem.*, **209,** 377 (1954).
11. Van Slyke, D. D., and Rieben, W. K., *J. Biol. Chem.*, **156,** 743 (1944).
12. Folch, J., Lees, M., and Sloane-Stanley, G. H., *Federation Proc.*, **13,** 209 (1954).
13. Sperry, W. M., *Ind. and Eng. Chem, Anal. Ed.*, **14,** 88 (1942).
14. Sperry, W. M., and Webb, M., *J. Biol. Chem.*, **187,** 97 (1950).
15. Van Slyke, D. D., and Sacks, J., *J. Biol. Chem.*, **200,** 525 (1953).
16. Christensen, H. N., *J. Biol. Chem.*, **129,** 531 (1939).
17. Folch, J., and Van Slyke, D. D., *J. Biol. Chem.*, **129,** 539 (1939).
18. Sobel, A. E., Mayer, A. M., and Gottfried, S. P., *J. Biol. Chem.*, **156,** 355 (1944).
19. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and LeBaron, F. N., *J. Biol. Chem.*, **191,** 833 (1951).

A GLUTATHIONE REDUCTASE FROM ESCHERICHIA COLI*

By ROBERT E. ASNIS

(From the Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania)

(Received for publication, July 26, 1954)

Earlier studies had demonstrated that a flavoprotein capable of mediating a triphosphopyridine nucleotide (TPN)-linked reduction of Furacin (5-nitro-2-furaldehyde semicarbazone) was present in cell-free extracts of Furacin-susceptible strains of *Escherichia coli*, but was absent in extracts of resistant strains (1). Dr. Philip E. Hartman, who was then associated with our department, suggested to us that the flavoprotein involved might be a TPN-linked reductase for oxidized glutathione (GSSG). The presence of such a GSSG reductase had already been demonstrated in cell-free extracts of yeast (2), plant tissues (3–5), mammalian tissues (6), and Neurospora (7), although these reductases had not been identified specifically as flavoproteins.

A partially purified preparation of our Furacin-reducing flavoprotein was indeed found to contain a very active TPN-linked GSSG reductase. However, it quickly became evident that this reductase was a flavoprotein distinctly different from the Furacin-reducing one which we have been studying. Thus the latter flavoprotein was (*a*) absent in cell-free extracts of resistant strains, (*b*) after treatment with acid could be almost completely reactivated by 3×10^{-6} M riboflavin phosphate (FP) but not by an equivalent concentration of flavin adenine dinucleotide (FAD), and was (*c*) completely destroyed by being heated for 10 minutes at 70°. The GSSG reductase, on the other hand, was (*a*) present in amounts with high activity in extracts of both susceptible and resistant strains of *E. coli*, (*b*) could be reactivated after acid treatment by 3×10^{-6} M FAD, but not by as much as 50 times this concentration of FP, and (*c*) lost only 10 to 15 per cent of its activity when heated to 70° for 10 minutes. Since such treatment enabled us to achieve a relatively high degree of separation of the GSSG reductase, some further characterization studies of this enzyme were made and are here presented.

Test Methods and Materials

Test System—Enzymatic activity was measured with a Beckman spectrophotometer, model DU, in terms of the rate of decrease of optical density

* This work was made possible by grants from the Eaton Laboratories, Inc., Norwich, New York.

at the 340 mμ absorption peak for reduced TPN (TPNH). Unless otherwise specified, all the studies were carried out with final concentrations of 3.6×10^{-5} M TPNH, 5.4×10^{-4} M GSSG, and M/15 phosphate buffer, pH 7.0, in a total volume of 3.0 ml. at 30°. Control systems containing TPNH and enzyme but no GSSG showed no activity.

Preparation of Enzyme—Crude cell-free extracts of *E. coli*, strain ECFS, were prepared as previously described for the partial purification of glycerol dehydrogenase up to the step at which the extracts used to prepare the dehydrogenase were heated to 60–63° for 90 minutes (8).

At this step removal of most of the nucleoproteins was accomplished by the following modification of a method described by Korkes and his coworkers (9). A volume of 1.0 M manganous chloride equal to 0.05 the volume of the crude extract was added slowly to it at 3–5° during constant stirring, and the extract was held at 3–5° for an additional 30 minutes with frequent stirring. The precipitated material was then removed by centrifugation for 20 minutes at 10,000 r.p.m. ($12,800 \times g$) in a Servall angle centrifuge, and the supernatant fluid was dialyzed overnight at 3–5° against about 15 times its volume of distilled water. Further precipitation occurred during this dialysis. Solid ammonium sulfate was added to the dialyzed material to 40 per cent saturation, and the precipitate was removed by centrifugation in the Servall centrifuge for 20 minutes at 10,000 r.p.m. and discarded. The supernatant solution was now brought to 60 per cent saturation with solid ammonium sulfate and centrifuged as before and the supernatant liquid discarded. The precipitate was dissolved in a volume of distilled water equal to half the original volume of the extract, heated to 70° for 10 minutes in a water bath, and rapidly chilled to 3°, and the heat-coagulated protein was removed by centrifugation as described above. Solid ammonium sulfate was added to the supernatant fluid to 60 per cent saturation at 3° and the material recentrifuged. The precipitate was again dissolved in a volume of cold (3–5°) distilled water equal to one-half the volume of the original crude extract. This solution was then dialyzed twice for 4 hours at 3–5° against a solution containing 0.1 M phosphate buffer, pH 7.0, and 0.001 M cysteine 30 times its volume.

The above procedure resulted in an approximately 50-fold purification of the enzyme in terms of TPNH oxidized per mg. of protein nitrogen. The best preparations had a specific activity of 2.6 in terms of micrograms per ml. of total protein required to produce a decrease of 0.100 in the optical density reading of the spectrophotometer at 340 mμ in the 1st minute. Such partially purified preparations did not oxidize either reduced diphosphopyridine nucleotide (DPNH) or TPNH at pH 7.0 in the presence of cystine, O_2, triphenyltetrazolium chloride, neotetrazolium chloride, cytochrome *c*, or Furacin. In the presence of TPNH, sodium 2,6-dichloro-

benzenoneindophenol was reduced at a measurable but relatively slow rate. There was no measurable oxidation of DPNH in the presence of enzyme and GSSG.

These preparations were quite stable and could be kept in the refrigerator at 4° for several weeks with only slight loss of activity. They were best preserved in the frozen state, since freezing resulted in no loss of activity.

It should be noted here that omission of glucose from the media in which the *E. coli* cells were grown greatly decreased the amount of GSSG reductase present in the cell-free extracts. Thus extracts of cells grown in media without glucose had 50 to 60 per cent less GSSG reductase activity. Interestingly enough, there was no measurable decrease in the amount of the previously mentioned TPN-linked Furacin-reducing flavoprotein in extracts of cells grown without glucose.

Acid Treatment of GSSG Reductase was carried out by the following modification of the method of Warburg and Christian (10). A given volume of the flavoprotein was chilled to 1° and held at this temperature. There was added during constant stirring an equal volume of cold (3°) saturated ammonium sulfate solution which had been acidified to pH 0.6 with concentrated hydrochloric acid. This preparation was held at 1° and vigorously stirred for 10 minutes, after which it was centrifuged for 10 minutes at 10,000 r.p.m. (10,500 × *g*) in a Spinco refrigerated centrifuge. The supernatant fluid was discarded and the precipitate redissolved in a volume of cold (3°) M/15 phosphate buffer, pH 7.0, equal to the original volume of enzyme preparation used.

TPNH was prepared from 80 per cent TPN "80" (Sigma). For most of the experiments the TPN was reduced with hydrosulfite by the following modification of the method of Ohlmeyer for reduction of DPN (11). 10 mg. of TPN were dissolved in 5 ml. of a solution containing 1.0 per cent sodium bicarbonate and 0.1 per cent sodium hydrosulfite. This solution was allowed to stand for 2 hours, after which it was vigorously aerated for 30 minutes. By this method 90 to 95 per cent of the theoretical yield of TPNH was obtained.

Such chemically reduced TPN was used in all of the experiments except those involving iodometric titration of GSH and the reduction of sodium 2,6-dichlorobenzenoneindophenol. Apparently traces of either sodium hydrosulfite or sodium sulfite present in these TPNH preparations caused a non-enzymatic reduction of iodine and of sodium dichlorobenzenoneindophenol. Therefore, in all experiments involving these two reagents, TPNH was prepared enzymatically with glucose-6-phosphate and a preparation of glucose-6-phosphate dehydrogenase. After enzymatic reduction of TPN was complete, the dehydrogenase was inactivated by heating of the preparation in a boiling water bath for 5 minutes.

XUM

DPNH was prepared from chromatographically pure DPN (Schwarz Laboratories) by the hydrosulfite method described for TPNH.

GSSG and GSH (sodium salt) were commercial preparations.

FAD used in preliminary experiments was a 15 per cent preparation, FAD "15" (Sigma). For the final experiment, shown in Fig. 4, Dr. D. J. O'Kane kindly gave us some 80 per cent FAD, which had been prepared in the laboratories of Dr. I. C. Gunsalus.

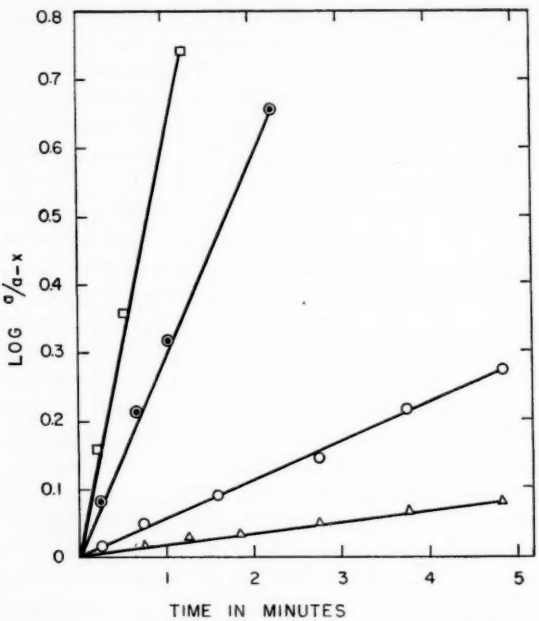


FIG. 1. First order reaction rates of the TPN-linked enzymatic reduction of GSSG. a = initial concentration of TPNH, x = decrease in concentration of TPNH in a given time, □ = 4.6 γ per ml. of final total protein concentration, ◉ = 2.3 γ per ml. of final total protein concentration, ○ = 1.15 γ per ml. of final total protein concentration, △ = 0.58 γ per ml. of final total protein concentration.

FP was a commercial synthetic preparation.

Quantitative determination of GSH was made by titration with 0.002 N iodine in acid solution (3).

Protein Determination—The protein content of the cell-free enzyme preparations was estimated turbidimetrically after precipitation with trichloroacetic acid (12).

Results

Enzymatic Activity—In the presence of GSSG the enzyme mediated oxidation of TPNH at a rate indicative of a first order reaction (Fig. 1). That the rate of oxidation of TPNH is a function of enzyme concentration is illustrated in Fig. 2. Stoichiometrically an approximate 1:2 molar rela-

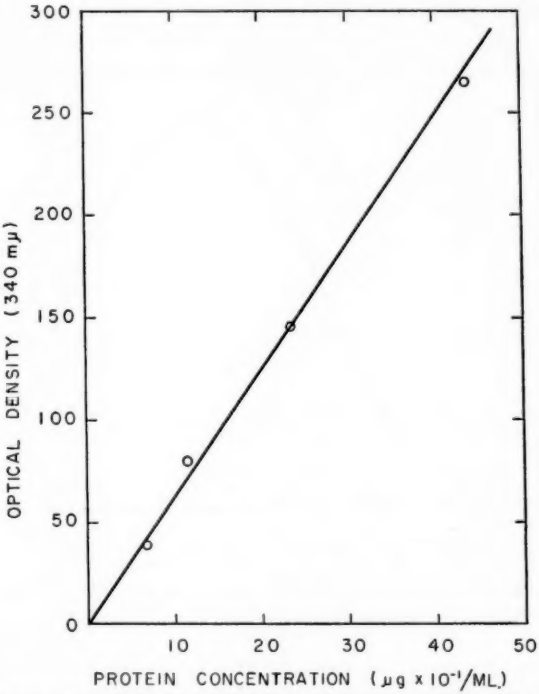


FIG. 2. Relationship of enzyme concentration in terms of final protein concentration. Optical density readings taken 1 minute after the start of each reaction.

tionship between TPNH oxidized and GSH produced was demonstrated by the following: TPNH oxidized, 0.708, 0.375, 0.927 μmole; GSH produced, 1.608, 0.790, 2.143 μmoles, respectively, by titration with 0.002 N I_2. This is in agreement with the schematic equation

$$TPNH + H^+ + GSSG \rightleftharpoons TPN^+ + 2GSH$$

The concentrations for optimal activity ($V_{max.}$) were approximately 1.2×10^{-4} mole per liter for TPNH and approximately 3.3×10^{-3} mole

per liter for GSSG. The K_s for the reductase and TPNH was found to be 3.7×10^{-5} mole per liter; the K_s for the reductase and GSSG was found to be 1.4×10^{-3} mole per liter. As previously reported (3, 6), the apparent equilibrium point of the reaction is greatly in favor of the formation of GSH. Thus when final concentrations of 4.3×10^{-4} M GSH and 6.7×10^{-5} M TPN were used over a pH range from 6.0 to 10.0, there was never any detectable reduction of TPN.

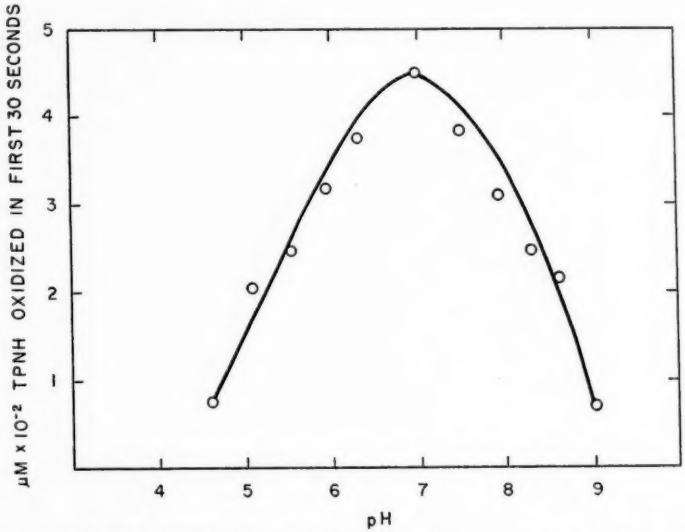


FIG. 3. Relationship of GSSG reductase activity to pH. Optical density readings taken 1 minute after the start of the reaction.

Biochemical Properties of Enzyme—The pH range for activity and the pH optimum of approximately 6.9 are shown in Fig. 3.

That the enzyme is a flavoprotein is evident from its inactivation by acid treatment and subsequent reactivation by the addition of FAD (Fig. 4). That FAD is probably the natural prosthetic group is indicated by the fact that resolved preparations could not measurably be reactivated by molar concentrations of FP or riboflavin 50 times the active concentration of FAD used (Fig. 4). It should be noted here that there was no measurable non-enzymatic oxidation of TPNH at pH 7.0 in the presence of GSSG and 3×10^{-6} M FAD. By concentrating the total yield of partially purified flavoprotein from 24 liters of culture into 3.0 ml., it was possible to analyze it spectrophotometrically. There was strong absorption in the

ultraviolet region with a maximum at 267 to 268 mμ. In the visible region there was only one small but unmistakable maximum at 450 mμ.

The effect of some familiar enzyme inhibitors and three antibiotics on the reductase was investigated, and the results are shown in Table I. As can

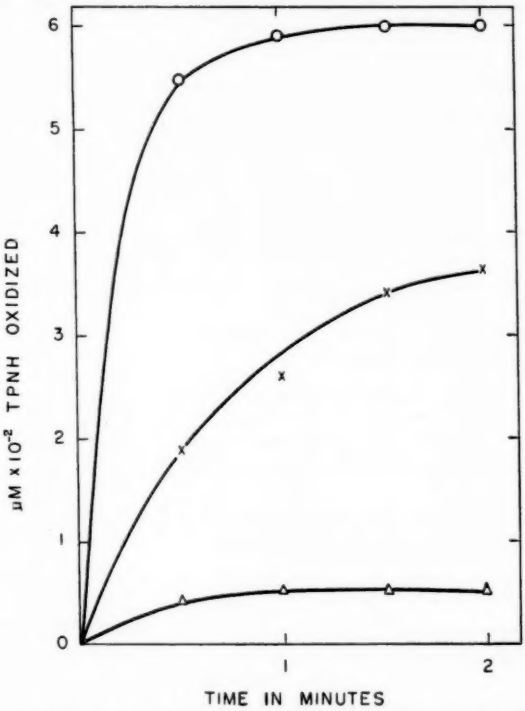


FIG. 4. Reactivation of acid-treated reductase by 3×10^{-6} M FAD. ○ = untreated enzyme, × = acid-treated enzyme + 3×10^{-6} M FAD, △ = acid-treated enzyme, △ = acid-treated enzyme + 1.5×10^{-4} M FP, △ = acid-treated enzyme + 1.5×10^{-4} M riboflavin.

be seen, the reductase was relatively resistant to the effects of several metals known to inhibit certain enzymes and was highly resistant to cyanide (KCN) and to the antibiotics used. Sodium *p*-chloromercuribenzoate completely inhibited it in a final concentration of 0.0001 M; this inhibition could be completely reversed by the addition of 0.001 M cysteine, indicating that the reductase is dependent on sulfhydryl groups for its activity.

There was no evidence for the presence of any essential metal component

in the reductase. Thus no loss in activity occurred when 5.0 ml. of the partially purified preparation were dialyzed overnight at 3–5° against 1000 ml. of a solution containing 0.1 M phosphate buffer, pH 8.2, 0.01 M KCN, and 0.001 M cysteine. Moreover, as can be seen in Table I, 0.1 M KCN (a strong metal-binding agent) had no measurable inhibitory effect on the enzyme.

As already indicated in the method for its partial purification, the reductase is relatively heat-stable. When held at 65° for 10 minutes it showed no loss in activity. 10 minutes at 70° caused only a 10 to 15 per cent loss of activity. However, 10 minutes at 80° resulted in complete loss of activity.

TABLE I

Effect of Various Enzyme Inhibitors and Antibiotics on GSSG Reductase Activity

Compound tested	Final molar concentration of compound	Per cent inhibition
Sodium *p*-chloromercuribenzoate	0.0001	100
" "	0.00001	20
Cu^{++} ($CuSO_4$)	0.001	100
" "	0.0001	45
Zn^{++} ($ZnSO_4$)	0.001	50
Fe^{+++} ($FeCl_3$)	0.001	50

KCN (0.1 M), penicillin (0.01 M), Chloromycetin (0.0001 M), Aureomycin (0.00001 M), and Furacin (0.0001 M) showed no inhibition.

DISCUSSION

The presence of a highly active GSSG reductase in cell-free extracts of *E. coli* is not surprising in view of its wide distribution, as shown by earlier investigators (2–7). It is even less surprising in the light of recent studies demonstrating that cells of *E. coli* contain large amounts of GSH (13). GSH apparently has many functions in life processes (14–16).

That the GSSG reductase has proved to be a flavoprotein is in agreement with the fact that many other enzymes mediating the oxidation of reduced pyridine nucleotides have proved to be flavoproteins, such as, for example, the diaphorases, the cytochrome reductases, and nitrate reductase.

The author wishes to express his appreciation to Dr. Philip E. Hartman for advice, and to Miss Mary Catherine Glick and Miss Ute Busemann for technical assistance.

SUMMARY

1. A highly specific, relatively heat-stable TPN-linked GSSG reductase has been isolated from a strain of *E. coli* and partially purified.

2. Reduction of 1 mole of GSSG to 2 moles of GSH for every mole of TPNH oxidized has been demonstrated.

3. The GSSG reductase has been shown to be a flavoprotein, for which FAD (but not FP or riboflavin) can serve as the prosthetic group.

4. Kinetic studies of the partially purified enzyme showed the constant K_s for TPNH and for GSSG to be 3.7×10^{-5} and 1.4×10^{-3} mole per liter, respectively.

5. The optimal activity was at pH 6.9. The enzyme was 100 per cent inhibited by 0.0001 M *p*-chloromercuribenzoate and this inhibition was completely reversed by 0.001 M cysteine, indicating dependence of the enzyme on sulfhydryl groups for its activity. In relatively high (0.001 M) concentrations, Fe^{+++} and Zn^{++} caused 50 per cent inhibition; Cu^{++} at 0.001 and 0.0001 M caused 100 and 45 per cent inhibition, respectively. KCN (0.1 M), penicillin (0.01 M), Chloromycetin (0.0001 M), Aureomycin (0.00001 M), and Furacin (0.0001 M) caused no measurable inhibition.

BIBLIOGRAPHY

1. Asnis, R. E., *Bact. Proc.*, 81 (1953).
2. Meldrum, N. U., and Tarr, H. L. A., *Biochem. J.*, **29**, 108 (1935).
3. Mapson, L. W., and Goddard, D. R., *Biochem. J.*, **49**, 592 (1951).
4. Conn, E. E., and Vennesland, B., *J. Biol. Chem.*, **192**, 17 (1951).
5. Anderson, G. A., Stafford, H. A., Conn, E. E., and Vennesland, B., *Plant Physiol.*, **27**, 675 (1952).
6. Rall, T. W., and Lehninger, A. L., *J. Biol. Chem.*, **194**, 119 (1952).
7. Nicholas, D. J. D., Nason, A., and McElroy, W. D., *J. Biol. Chem.*, **207**, 341 (1954).
8. Asnis, R. E., and Brodie, A. F., *J. Biol. Chem.*, **203**, 153 (1953).
9. Korkes, S., del Campillo, A., Gunsalus, I. C., and Ochoa, S., *J. Biol. Chem.*, **193**, 721 (1951).
10. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 368 (1938).
11. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
12. Stadtman, E. R., Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **191**, 365 (1951).
13. Roberts, R. B., and Bolton, E. T., *Science*, **115**, 479 (1952).
14. Racker, E., and Krimsky, I., *Nature*, **169**, 1043 (1952).
15. Barron, E. S. G., *Texas Rep. Biol. and Med.*, **11**, 653 (1953).
16. Racker, E., *Federation Proc.*, **12**, 711 (1953).

XUM

THE ESTIMATION OF HYDROXYSTEROIDS*

By BILLY BAGGETT, LEWIS L. ENGEL, AND LINDA L. FIELDING

(From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts)

(Received for publication, August 19, 1954)

A number of methods have been described for the quantitative estimation of steroids having rather specific groups, *e.g.* 17-ketone groups, or α-ketolic side chains at C-17. Procedures with such high specificity have been extremely useful in studies of steroid metabolism. They have been particularly valuable for the estimation of such compounds in urinary extracts and in the fractionation of these extracts. There are, however, steroids present in such extracts which, lacking these specific groupings, are not measured by any of the commonly employed methods. One large class of compounds of this type is that consisting of non-ketonic steroid alcohols. Over twenty compounds of this type have been isolated from urine, and included in this group are metabolites of androgens and adrenal cortical steroids as well as the major recognized metabolite of progesterone, which is a non-ketonic steroid alcohol, pregnane-3α,20α-diol (1). A method for the quantitative estimation of such hydroxysteroids should prove useful for the evaluation of the function of the endocrine organs and in systematic fractionations of this group of metabolites.

Several methods have previously been described for the estimation of non-ketonic steroid alcohols (1–8) and have been applied to urinary extracts (9–13). The results of these relatively few studies indicate that further investigation of this fraction should prove profitable.

The method reported in this paper is based on the formation of acetate esters of the steroid hydroxyl groups, followed by the conversion of the acetates to acetohydroxamic acid with alkaline hydroxylamine. The acetohydroxamic acid forms a purple complex with ferric ion. The intensity of the resulting color is then used as a measure of the original con-

* This work was supported by grants from the National Cancer Institute, United States Public Health Service (grant No. C-1393(C,C2)), the American Cancer Society upon recommendation by the Committee on Growth of the National Research Council, the Jane Coffin Childs Memorial Fund for Medical Research, and an Institutional Grant from the American Cancer Society to the Massachusetts General Hospital. Preliminary reports of portions of this work were presented at the Laurentian Hormone Conference, Shrewsbury, Massachusetts, May 31–June 4, 1953 (1), and the Thirty-eighth meeting of the American Society of Biological Chemists, Atlantic City, April 12–16, 1954. This is Publication No. 832 of the Cancer Commission of Harvard University.

XUM

tent of hydroxyl groups. Quantitative methods based on the formation of hydroxamic acids have been reported for the estimation of anhydrides such as acetyl phosphate (14), glycerol esters (15–17), water-soluble esters (18), and amides (19). The use of such a method for hydroxyl groups in lipides was suggested by Hill (15), who did some preliminary work on the problem. Recently Zaffaroni has reported the utilization of these reactions for the determination of 11-deoxycorticosterone acetate and has suggested the more general use for steroid alcohols (20).

The experiments described here serve to elucidate the optimal conditions for the application of these reactions to the estimation of hydroxysteroids. The resulting method has been applied to a number of pure steroid alcohols and to urinary extracts. The results of these studies are presented.

EXPERIMENTAL

Three reactions are involved in the estimation of hydroxysteroids by the method described here. The first is the acetylation of the hydroxyl groups. The second is the cleavage of the acetoxyl groups with hydroxylamine to form acetohydroxamic acid. The third reaction is the formation of the purple ferric ion-acetohydroxamic acid complex. Each of these reactions will be considered separately.

Acetylation of Hydroxysteroids—Only one basic method for the acetylation of steroid hydroxyl groups has been investigated, the pyridine-catalyzed reaction with acetic anhydride. Relatively few variations of this method have been studied, since quantitative acetylation with this reagent is not difficult and can be achieved under a variety of conditions. The reagent used throughout this work has been a freshly prepared mixture of equal volumes of acetic anhydride and pyridine. Quantitative acetylation, determined by conversion to acetohydroxamic acid as described later, was achieved in 1 hour on a boiling water bath with 0.2 ml. of this reagent for 0.5 to 5 μeq. of hydroxysteroid (0.2 to ? mg. of cholesterol). Most hydroxyl groups studied were quantitatively esterified in 10 minutes; however, the 3β-hydroxyl group of coprostanol required 60 minutes. Hence, the longer period has been chosen for routine use. It has been found, in agreement with the results of others, that pyridine-acetic anhydride fails to acetylate 11β-hydroxyl or tertiary 17α-hydroxyl groups.

Preliminary studies have indicated that it may be possible to acetylate certain groups selectively by varying the conditions for acetylation.

Conversion of Acetoxyl Groups to Acetohydroxamic Acid—The basic reagent used for this reaction is a solution of hydroxylamine in alcoholic potassium hydroxide. This is prepared by mixing alcoholic KOH with concentrated aqueous hydroxylamine hydrochloride and centrifuging to remove precipitated KCl. The effects of variations in solvent, concentra-

tion of KOH, concentration of hydroxylamine, and reaction time have been investigated with cholesteryl acetate, 11-deoxycorticosterone acetate, and allopregnane-3β,16α,20β-triol triacetate as model compounds.

Solvent—Aqueous methanol and aqueous ethanol have been used as solvents for this step of the procedure; however, ethanol is preferable, since most of the compounds studied are more readily soluble in ethanol than in methanol. The concentration of water in the ethanol has been varied from 3 to 15 per cent without significant effect on the course of the reaction. Therefore, the concentration has been kept to the minimum necessary to contain the hydroxylamine hydrochloride added in preparation of the reagent. It has been found that dissolving the steroid in a minute volume of diethyl ether before adding the alcoholic hydroxylamine-potassium hydroxide reagent greatly facilitates the solution of the steroid acetates in the reagent.

Concentrations of KOH and NH_2OH—The effects of varying the concentrations of KOH and NH_2OH independently have been studied. It has been found that the rate of the reaction is increased by increases in KOH concentration. Increases in NH_2OH concentration lead to increased stability of the acetohydroxamic acid. Pure acetohydroxamic acid does not undergo measurable decomposition in 10 minutes in the reagent finally selected for use and described below. When the hydroxylamine concentration is decreased by 50 per cent, 20 per cent of pure acetohydroxamic acid is destroyed in the same period of time at a rate independent of the concentration of alkali. Extensive studies of the effects of variations in the concentrations of the two reagents were not necessary. Reagents were initially tried which contained lower concentrations of hydroxylamine and potassium hydroxide. The concentrations were then increased progressively by large increments, which led to increased rates of reaction and stability of the hydroxamic acid. The concentrations finally selected for the reagent were those which gave quantitative yields of hydroxamic acid in a reasonable period of time, 10 minutes. No advantage could be foreseen in the use of higher concentrations.

Formation of Ferric-Acetohydroxamic Acid Complex—The final step in the series of reactions constituting the determination of hydroxyl groups is the formation of the colored complex of the hydroxamic acid with ferric ion. In the actual procedure, it is necessary to neutralize the potassium hydroxide from the preceding step, since the complex is stable over a relatively narrow pH range, 1.0 to 1.4 (18). The optimal amounts of acid and ferric chloride were determined empirically and are contained in the reagent described below. Lower concentrations of ferric chloride fail to yield maximal color with acetohydroxamic acid, and higher concentrations lead to unnecessarily high blanks. The concentration of hydrochloric

XUM

acid is that found to yield optimal color intensity and stability. Variations of up to 5 per cent in the concentration of hydrochloric acid are without measurable effect, but greater variations in either direction markedly decrease the color intensity of the complex.

Hill suggested the use of ferric perchlorate in place of ferric chloride and either perchloric or nitric acid along with the hydrochloric acid (15, 16). These substitutions were tried and did not in any way improve the complex formation under the conditions used here. Likewise the separate addition of the acid and ferric chloride did not offer any advantage.

Procedure for Estimation of Hydroxysteroids

The experiments outlined above have served to define conditions necessary for quantitative results in each step of the procedure. The next portion of the paper will describe the method for estimating hydroxysteroids which has resulted from the utilization of the information gained from the studies of these reactions.

Reagents—Acetic anhydride. Mallinckrodt, analytical reagent, redistilled; b.p. 137–138°.

Anhydrous pyridine. Merck, reagent, or Mallinckrodt, analytical reagent, refluxed over BaO and redistilled; b.p. 114°.

Acetic anhydride-pyridine reagent. Prepared by mixing equal volumes of acetic anhydride and anhydrous pyridine immediately before use.

Methanol, C. P. National Aniline.

Diethyl ether. Mallinckrodt, analytical reagent.

Hydroxylamine hydrochloride solution. Prepared by dissolving 56 gm. of hydroxylamine hydrochloride (Eastman or Matheson) in 100 ml. of distilled water.

Alcoholic potassium hydroxide solution. Prepared by dissolving 15.8 gm. of potassium hydroxide pellets (Mallinckrodt, analytical reagent, 85 per cent KOH) in 100 ml. of 95 per cent ethanol. Stable for at least 1 month when kept at 5°.

Alkaline hydroxylamine reagent. Prepared by mixing 7.5 volumes of alcoholic potassium hydroxide solution and 1 volume of hydroxylamine hydrochloride solution and centrifuging to remove the precipitate. Must be prepared immediately (not more than 15 minutes) before use.

Ferric chloride-hydrochloric acid reagent. Prepared by dissolving 1.7 gm. of $FeCl_3 \cdot 6H_2O$ (Mallinckrodt, analytical reagent) in 100 ml. of dilute HCl (6.5 ml. of concentrated HCl diluted to 100 ml. with H_2O).

Cholesterol standard. 1.00 mg. of cholesterol (m.p. 146–147°) per ml. in absolute ethanol.

Analytical Method—The samples to be analyzed, along with cholesterol standards, which should contain 0.5 to 5 μeq. of acylable hydroxyl, are ob-

tained dry in test-tubes calibrated for use as cuvettes in a photometer.[1] 0.2 ml. of the freshly prepared acetic anhydride-pyridine reagent is added to each tube, including empty ones to serve as blanks. The tubes are rotated to dissolve the samples, stoppered with corks wrapped in aluminum foil, and heated in a boiling water bath for 1 hour. The acetic anhydride and pyridine are then removed by evaporation under a stream of air in the water bath. When the odors of acetic anhydride and pyridine have disappeared, 0.1 ml. of methanol is added to each tube. The tubes are rotated to spread the methanol over the surface previously in contact with acetic anhydride and pyridine. The methanol is subsequently evaporated on a boiling water bath. This operation removes the last traces of the reagents.

Each acetylated sample is then dissolved in 0.2 ml. of diethyl ether, and 1 ml. of alkaline hydroxylamine reagent is added. The tubes are shaken and allowed to stand at room temperature for 10 minutes. 3 ml. of the ferric chloride-hydrochloric acid reagent are then added to each tube to neutralize the base and form the colored complex. Each tube must be stoppered and shaken immediately after this reagent is added to avoid reduction of ferric ion by alkaline hydroxylamine.[2]

At this point, the alcohol content of the solution is about 20 per cent; hence, most steroids and other lipides present will precipitate, causing cloudiness. This is overcome by adding 4 ml. of diethyl ether to each tube and shaking for a short period to extract the aqueous phase. This procedure, in addition to removing turbidity, extracts any lipide-soluble pigment from the aqueous solution. It is unnecessary to remove the ether layer, since it is above the light path of the photometer.

When 30 minutes have elapsed after the addition of the ferric chloride-hydrochloric acid reagent, the tubes are read in the photometer at 560 mμ.[3] The absorption spectrum for the colored ferric-acetohydroxamic acid complex was determined with the Cary spectrophotometer and the peak absorption found to be at 527 mμ. However, the peak is quite broad, and the absorption at 560 mμ is only slightly lower than at the peak. The blank color, due to ferric ion absorption, is below 400 mμ, but the absorption at 530 mμ is appreciable when the Coleman junior spectrophotometer

[1] Calibrated Pyrex test-tubes (13 × 100 mm.) were used in this work.

[2] Krogh-Keys syringe pipettes have been found useful for the addition of the ferric chloride-hydrochloric acid reagent, since delivery from these pipettes is accompanied by satisfactory mixing. The tubes are then stoppered and shaken after the reagent has been added to all tubes in a series.

[3] Immediately after adding the ferric chloride-hydrochloric acid reagent, erratic color formation occurs in many tubes, owing probably to the formation of some ferric hydroxide. This color fades quite rapidly after mixing and is completely absent after 30 minutes standing. The ferric-acetohydroxamic acid complex is reasonably stable under these conditions, fading at the rate of 2 per cent per hour.

XUM

is employed. The absorption is about 50 per cent lower at 560 mμ. For this reason, the latter wave-length has been used in the determinations. It is likely that a wave-length closer to the absorption maximum might be better with photometers having greater resolving power.

The readings of samples and standards are corrected for the blank reading, and the microequivalents of acylable hydroxyl per sample are calculated by comparison with standards of cholesterol[4] run at the same time.

Yields of Chromogen from Pure Steroids

Steroid Acetates—Cholesteryl acetate has been analyzed by the above method, with the omission of the acetylation steps. By comparison with pure acetohydroxamic acid, it was found to yield the ferric-acetohydroxamic acid chromogen quantitatively. Since cholesteryl acetate is readily available, it has been used as the standard against which other steroid acetates and steroid alcohols have been compared. The yields of chromogen from allopregnane-3β,16α,20β-triol triacetate and from deoxycorticosterone acetate were 91 per cent, compared with cholesteryl acetate on an equivalent basis. These low yields were increased by about 10 per cent upon acetylation. This indicates that the samples were not pure and had probably undergone some hydrolysis on standing. All three steroid acetates showed a linear relationship between weight and absorbance in the region studied, from 0.5 to 5 μeq.

Steroid Alcohols—A number of pure steroid alcohols have been subjected to the entire analytical procedure and their percentage yields of acetohydroxamic acid calculated on an equivalent basis by comparison with cholesteryl acetate. These compounds were selected to contain hydroxyl groups in the positions and with the configurations usually encountered in naturally occurring steroids. The yield of chromogen from each of these compounds is presented in Table I. All values are means of two or more sets of determinations carried out with at least two different levels of steroid, usually 1 and 3 μeq. The relationship between absorbance and quantity was found to be linear through the origin for all compounds studied. Statistical analysis of the data indicates that there is no significant difference between the yields of chromogen from the various compounds studied. The mean yield for all compounds studied is 96 per cent, with a standard deviation of ±3.7 per cent. The results on progesterone show that a hydroxyl group is necessary for chromogen formation.[5]

[4] As demonstrated below, all hydroxysteroids yield the same amount of chromogen per microequivalent of acylable hydroxyl; hence, any pure hydroxysteroid can be substituted for cholesterol as the standard.

[5] The source of the chromogen in this case is probably Δ^5-pregnen-3β-ol-20-one, a known contaminant of commercial progesterone (21).

Estimation of Hydroxysteroids in Neutral Urinary Extracts

In the application of the above method to neutral extracts of acid-hydrolyzed urine, it was found that these extracts, prior to acetylation, contain material which reacts with the alkaline hydroxylamine reagent to form products that subsequently yield chromogen with the ferric chloride reagent. This material presumably consists of esters, since it is substantially reduced by saponification of the extract with 1 N KOH in methanol prior to analysis, and since the extracts do not react with ferric chloride to form chromogen when the reaction with hydroxylamine is omitted. Investigation of the nature of this material is under way, since it may be

TABLE I

Percentage Yield of Acetohydroxamic Acid Chromogen from Various Steroids

Compound	Yield*
	per cent
Cholesterol	99
Coprostanol	94
Cholestanol	100
Epicoprostanol	98
Epicholestanol	95
Testosterone	94
Pregnane-3α,20α-diol	94
Allopregnane-3β,16α,20β-triol	97
Deoxycorticosterone	94
Progesterone	2†

* Based on comparison with the yield of chromogen from cholesteryl acetate.

† Calculated for a monohydroxy compound of molecular weight 314.

partly composed of esterified steroid alcohols, either excreted in this form or formed during the processing of the extracts. Until its nature is more clearly defined, however, it seems preferable to correct for its contribution to the final chromogen formation. This has been done routinely by running duplicate samples of the urinary extracts through the analytical procedure without the acetylation. The absorbance of this sample, referred to as the "preformed ester blank" is subtracted from the absorbance of the corresponding acetylated sample. This procedure also corrects for any pigment not extracted by the ether in the analytical procedure. The magnitude of this preformed ester blank is quite variable from extract to extract. In most cases it amounts to about 10 per cent of the value for the acylable material; however, in the extracts from a few individuals the values were considerably higher.

In the analysis of urinary extracts, the extracts, cholesterol standards,

XUM

and blanks are run through all steps of the analytical procedure as described above, beginning with the acetylation. Duplicate samples of the extracts along with unacetylated blanks are run through the steps beginning with the addition of 0.2 ml. of ether, the acetylation steps being omitted. The acetylated blank, referred to as the "acetylation blank," usually has an absorbance about 0.02 above that of the unacetylated blank, referred to as the "reagent blank." A difference greater than this indicates a lack of purity of either the pyridine or acetic anhydride.

TABLE II

Daily Excretion of Urinary Neutral Acylable Lipides

Sex	Age	Condition	Total
	yrs.		*μeq. per 24 hrs.*
F.	24	Normal	116
		Pregnancy (1st trimester)	194
		" (3rd ")	258
F.	24	Normal	162*
"	50	"	96
M.	53	"	150
"	68	Prostatic carcinoma	114
"	48	Carcinoma, breast	110
F.	55	" "	178
"	43	" "	49
M.	55	Rheumatoid arthritis	82
"	34	" "	125
F.	45	Addison's disease	26
M.	60	Adrenal carcinoma	410
F.	43	" "	288
"	21	Cushing's disease	177

* Mean of five values obtained at various phases of the menstrual cycle (range 133 to 184 μeq.).

To calculate the microequivalents of acylable hydroxyl in the unknown, the following formula is employed,

$$\mu eq_{\cdot unk.} = \frac{U - B_a - (B_{pe} - B_r)}{S - B_a} \times \mu eq_{\cdot std.}$$

where, $\mu eq_{\cdot unk.}$ = microequivalents of acylable hydroxyl in the unknown
U = absorbance of unknown at 560 mμ
B_a = " " acetylation blank at 560 mμ
B_{pe} = " " preformed ester blank at 560 mμ
B_r = " " reagent blank at 560 mμ
S = " " hydroxysteroid standard at 560 mμ
$\mu eq_{\cdot std.}$ = microequivalents of acylable hydroxyl in hydroxysteroid standard

In Table II are presented some representative results obtained by this method for the estimation of urinary neutral acylable lipides. These determinations were carried out in duplicate on aliquots of urinary extracts corresponding, in most cases, to about 1 per cent of a 24 hour urine specimen. The above formula was utilized for the calculations. These examples have been selected to demonstrate variations in the excretion of neutral acylable lipides with changes in the endocrine states of the patients.

Fig. 1 shows the effect of testosterone administration on the urinary excretion of neutral acylable lipides by a patient with rheumatoid arthritis.

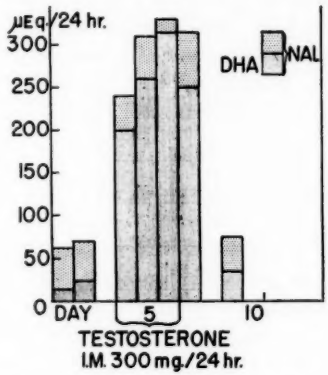


FIG. 1. The effect of intramuscular administration of testosterone on the urinary excretion of neutral acylable lipides (*NAL*) and ketosteroids in a male patient, 59 years old, with rheumatoid arthritis. *DHA* is the 17-ketosteroid value calculated as microequivalents of dehydroepiandrosterone per 24 hours. *NAL* represents the neutral acylable lipides as measured by the ferric-acetohydroxamic acid method.

The daily excretion of 17-ketosteroids (micromoles) as determined by a modification of the Zimmermann reaction (22, 23) is included for comparison. The administration of testosterone is accompanied by a marked increase in the excretion of materials measured by both methods; that is, 17-ketosteroids and neutral acylable lipides. The close correlation between the values for the two groups of compounds during administration of testosterone is consistent with the excretion of androsterone and etiocholan-3α-ol-17-one as the major urinary metabolites of this hormone.

DISCUSSION

The method presented here offers a simple and rapid method for the quantitative estimation of a group of urinary steroids not measured by more specific methods. Obviously this method has no intrinsic specificity

for steroid alcohols, but will likewise measure other lipide-soluble alcohols. However, the good correlation between the daily excretion of neutral acylable lipides and the state of endocrine balance suggests that under most conditions the major portion of the urinary neutral acylable lipides is composed of hydroxysteroids. The extent to which non-steroidal compounds contribute to the values obtained by this method must await the fractionation and identification of the components of this fraction. Studies in this direction are now under way.

This method has several potential uses (1) in addition to its application for the estimation of urinary neutral acylable lipides. It has been employed in this laboratory for the determination of the number of acylable hydroxyl groups in pure compounds of unknown structure in which the approximate molecular weights were known. It has also been used to follow the course of hydrolysis of acetate esters of hydroxysteroids.

SUMMARY

1. A method is presented for the quantitative estimation of acylable hydroxyl groups in lipides.

2. The method has been applied to steroids possessing hydroxyl groups in the positions and with the configurations commonly encountered in urinary steroids. The yield of chromogen has been found to correspond to 96 per cent of the theoretical yield for all hydroxyl groups acetylated under base catalyzed conditions.

3. The urinary excretion of neutral acylable lipides has been found to correlate with the state of endocrine function, suggesting that the compounds measured are predominantly steroids.

The authors wish to express their thanks to the following for their generous gifts of compounds: Ciba Pharmaceutical Products, Inc. (deoxycorticosterone acetate, progesterone, and testosterone), Dr. John D. Gregory (acetohydroxamic acid), Parke, Davis and Company (pregnanediol).

We also wish to express our thanks to Miss Gladys Ekman for the ketosteroid analyses and to Miss Electra Paskalides for the preparation of the urinary extracts.

BIBLIOGRAPHY

1. Engel, L. L., and Baggett, B., *Recent Progress Hormone Res.*, **9,** 251 (1954).
2. Pincus, G., *Endocrinology*, **35,** 227 (1944).
3. Dobriner, K., Lieberman, S., and Rhoads, C. P., *J. Biol. Chem.*, **172,** 241 (1948).
4. Lieberman, S., Fieser, L. F., Dobriner, K., and Rhoads, C. P., *Endocrinology*, **35,** 223 (1944).
5. Tompsett, S. L., and Oastler, E. G., *Glasgow Med. J.*, **29,** 133 (1948).

6. Engel, L. L., Patterson, H. R., Wilson, H., and Schinkel, M., *J. Biol. Chem.*, **183,** 47 (1950).
7. Kellie, A. E., Smith, E. R., and Wade, A. P., *Biochem. J.*, **53,** 578 (1953).
8. Nosek, J., Krestýnová, O., and Podivinský, R., *Proc. Sborník Mezinárod. Polarog. Sjezdu Praze*, pt. 3, 624 (1951); *Chem. Abstr.*, **47,** 11037 (1953).
9. Engel, L. L., Proceedings of 1st clinical ACTH conference, 166 (1950).
10. Nathanson, I. T., Engel, L. L., and Kelley, R. M., Proceedings of 2nd clinical ACTH conference **1,** 54 (1951).
11. Nathanson, I. T., Engel, L. L., Kelley, R. M., Ekman, G., Spaulding, K. H., and Elliott, J., *J. Clin. Endocrinol. and Metabolism*, **12,** 1172 (1952).
12. Kellie, A. E., and Wade, A. P., *Biochem. J.*, **53,** 582 (1953).
13. Kellie, A. E., and Wade, A. P., *Brit. Med. J.*, **2,** 594 (1953).
14. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159,** 21 (1945).
15. Hill, U. T., *Ind. and Eng. Chem., Anal. Ed.*, **18,** 317 (1946).
16. Hill, U. T., *Anal. Chem.*, **19,** 932 (1947).
17. Bauer, F. C., Jr., and Hirsch, E. F., *Arch. Biochem.*, **20,** 242 (1949).
18. Hestrin, S., *J. Biol. Chem.*, **180,** 249 (1949).
19. Bergmann, F., *Anal. Chem.*, **24,** 1367 (1952).
20. Zaffaroni, A., *Recent Progress Hormone Res.*, **8,** 51 (1953).
21. Reich, H., Sanfilippo, S. J., and Crane, K. F., *J. Biol. Chem.*, **198,** 713 (1952).
22. Nathanson, I. T., and Wilson, H., *Endocrinology*, **33,** 189 (1943).
23. Wilson, H., and Nathanson, I. T., *Endocrinology*, **37,** 208 (1945).

XUM

URINARY METABOLITES OF ADMINISTERED CORTICOSTERONE

I. STEROIDS LIBERATED BY GLUCURONIDASE HYDROLYSIS*

By LEWIS L. ENGEL, PRISCILLA CARTER, AND LINDA L. FIELDING

(From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts)

(Received for publication, August 19, 1954)

While a number of urinary steroids related to the principal human adrenal secretory product, hydrocortisone, are known, little information is available regarding the metabolic fate of corticosterone. Mason (1) has isolated pregnane-3α,20α-diol-11-one from the urine of patients treated with dehydrocorticosterone. Lieberman, Fukushima, and Dobriner (2) have also isolated this substance, as well as pregnane-3α-ol-11,20-dione from human urine. In a recent publication, Gómez Mont and Berliner (3) reported the detection of corticosterone and an incompletely characterized tetrahydro derivative of dehydrocorticosterone in the urine of a patient treated with corticosterone. This report deals with the isolation and identification of allopregnane-3α,11β,21-triol-20-one, pregnane-3α,-11β,21-triol-20-one, pregnane-3α,21-diol-11,20-dione, and pregnane-3α,-20α-diol-11-one from the urine of a patient with rheumatoid arthritis who was treated with 300 mg. of corticosterone per day for 8 days.

Methods

Hydrolysis of Conjugates and Preparation of Extracts—One-tenth of each 24 hour specimen (equivalent to a total of eight-tenths of a 24 hour specimen) was boiled for 10 minutes (4), cooled, adjusted to pH 5.1 with acetate buffer, and treated with 30,000 Fishman units of beef liver β-glucuronidase (Warner). After incubation at 37° for 3 days, the hydrolysis mixture was extracted with methylene chloride and washed with saturated sodium bicarbonate solution and water. The residues obtained from these extracts by evaporation under reduced pressure were dissolved in ethanol.

* This work was supported by a grant from the National Cancer Institute, United States Public Health Service (grant No. C-1393(C,C2)), an allocation from an Institutional Grant from the American Cancer Society to the Massachusetts General Hospital, a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council, and a grant from the Jane Coffin Childs Memorial Fund for Medical Research. A preliminary report was presented at the meeting of the American Society of Biological Chemists, Atlantic City, April 12–16, 1954. This is Publication No. 833 of the Cancer Commission of Harvard University.

XUM

Assay of Formaldehydogenic Lipide—Aliquots were withdrawn from ethanol solutions of the extracts and analyzed for formaldehydogenic lipides by a modification of the method of Hollander *et al.* (5). The ethanol solution of the sample was transferred into the outer well of a Conway unit and the solvent allowed to evaporate spontaneously. The residue was dissolved in 0.5 ml. of triethyl phosphate. 2 ml. of 0.30 per cent chromotropic acid in 15 M sulfuric acid were placed in the inner well, and finally 1 ml. of 0.02 M aqueous sodium periodate solution in 0.15 M H_2SO_4 was added to the outer well. The units were then sealed and oxidation and diffusion were allowed to proceed at room temperature in the dark for 16 hours. The contents of the inner wells were then transferred to 13 × 100 mm. Pyrex test-tubes calibrated for use as cuvettes, heated on the boiling water bath for 30 minutes, cooled, and the absorbance at 570 mμ measured in a Coleman junior spectrophotometer.

Separation of Ketonic and Non-Ketonic Fractions—This separation was accomplished in the usual manner with Girard's Reagent T (6). The reaction was carried out at room temperature for 24 hours, and all extractions were made with chloroform.

Counter-Current Distributions—A 100 tube glass instrument (7) was used for all distributions. The solvents were mutually saturated, and the system (50 per cent cyclohexane-50 per cent ethyl acetate/30 per cent ethanol-70 per cent water) used was selected so that hydrocortisone would have $K = 1.1$ (8). Analyses for formaldehydogenic lipides were performed on selected fractions.

Paper Chromatography—Fractions and appropriate standards were applied to 7 inch strips of Whatman No. 1 filter paper and chromatographed in the toluene-propylene glycol system of Burton, Zaffaroni, and Keutmann (9). The zones were detected by examination under ultraviolet light and by spraying test strips with alkaline blue tetrazolium. The steroids were eluted with methanol, and the extracts evaporated to dryness, dissolved in chloroform, and washed with small volumes of water to remove propylene glycol. Prior to examination in the infra-red, the fractions eluted from paper chromatograms were further purified. The samples were dissolved in a small volume of cyclohexane-ethyl acetate and applied to a 7 × 150 mm. column of silica gel (Davison, 200 to 300 mesh). Elution was carried out with cyclohexane containing increasing amounts of ethyl acetate and finally with ethyl acetate.

Acetylation—The sample was dissolved in 0.1 ml. of pyridine (distilled from barium oxide) and treated with 0.1 ml. of redistilled acetic anhydride. After standing overnight at room temperature, the volatile reagents were removed at about 60° in a stream of air. The last traces were eliminated by storage overnight in a vacuum desiccator over silica gel.

Chromic Acid Oxidation—Chromic acid oxidation was carried out as described by Zaffaroni and Burton (10).

Preparation of Methyl Alloetianates—The sample was dissolved in 1 ml. of redistilled methanol and treated with 4 per cent periodic acid in 0.2 N H_2SO_4 and allowed to stand at room temperature overnight. The reaction mixture was then diluted with 10 ml. of water and extracted with five 10 ml. portions of chloroform and the extract washed with five 5 ml. por-

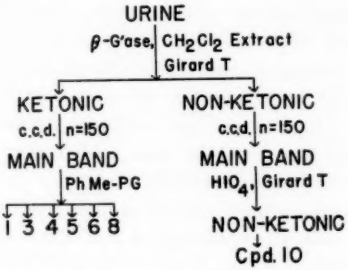


FIG. 1. Fractionation of urine for the isolation of corticosterone metabolites

TABLE I

Paper Chromatography of Ketonic Fraction

Zone No.*	Blue tetrazolium	Formaldehydogenic lipides†
1	+	0.3
3	+	1.1
4	+	2.3
5	+	13.2
6	+	5.6
8	+	0.1

* The zones are listed in order of decreasing mobility.

† Calculated as mg. of deoxycorticosterone.

tions of 0.1 M sodium carbonate. The alkaline extracts were adjusted to pH 2 and extracted with five 10 ml. portions of chloroform. The chloroform extracts were washed twice with 10 ml. of water and evaporated to dryness under diminished pressure.

The residue was esterified with freshly distilled ethereal diazomethane prepared from *N*-methyl-*N*-nitroso-*N'*-nitroguanidine[1] (11). The crude methyl esters were purified by passage through a column of silica gel as described above.

Infra-Red Spectra—A Perkin-Elmer double beam infra-red spectrometer

[1] Obtained from the Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

was used for all measurements. Unless otherwise indicated, the spectra were measured in carbon disulfide solution.

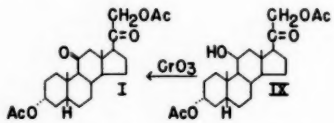
Results

The procedure employed for processing the urine is outlined in the flow sheet (Fig. 1). Both the ketonic and the non-ketonic fractions were subjected to 150 transfer counter-current distributions.

Analysis of the distribution of the ketonic fraction for formaldehydogenic lipides disclosed that the bulk of the material was contained in one broad band which extended from tubes 90 to 99 in the instrument and transfer numbers 150 to 110 in the portion withdrawn. Such a band spread was considerably greater than would be predicted for a single compound. Accordingly, this material was pooled and subjected to further fractionation by paper chromatography.

The results of paper chromatography are presented in Table I. Of the six zones observed, only three contained sufficient material for characterization.

Characterization of Zone 4—Examination of this material in the infra-red showed strong hydroxyl absorption and absorption in the carbonyl region strongly suggestive of the presence of 3-, 11-, or 20-carbonyl groups. Acetylation yielded a substance whose infra-red absorption was identical in every respect with that of an authentic specimen of pregnane-3α,21-diol-11,20-dione 3,21-diacetate (I).



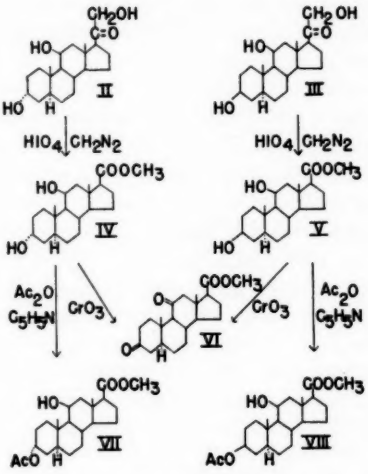
Characterization of Zone 5—The washed residue from Zone 5 crystallized spontaneously. After further purification by four crystallizations from aqueous methanol, 5 mg. of pure compound, m.p. 204–206° (uncorrected, Fisher-Johns) were obtained. This substance did not form an insoluble digitonide. Analysis for acylable hydroxyl groups (12) showed 1.8 hydroxyls for a molecular weight corresponding to a $C_{21}O_4$ structure.

The free compound was too sparingly soluble in either carbon disulfide or chloroform to yield satisfactory infra-red spectra. However, the diacetate had strong hydroxyl absorption and, in addition, bands at 1757 and 1735 cm.$^{-1}$, which indicated a 20-keto-21-acetoxy structure (13). This was in agreement with the observation that the free compound strongly reduced alkaline blue tetrazolium.

Chromic acid oxidation of the diacetate yielded a material which no

longer had hydroxyl absorption in the high frequency region. However, it retained its absorption at 1755 and 1735 cm.$^{-1}$ and possessed, in addition, a new band at 1711 cm.$^{-1}$. The presence of a hydroxyl group not susceptible to acetylation under conditions of basic catalysis and its ready oxidation to a ketone group absorbing at 1711 cm.$^{-1}$ make it apparent that the original compound must have contained an 11β-hydroxyl group.

Since the spectrum of the 11-ketodiacetate in the 1350 to 750 cm.$^{-1}$ region was distinctly different from that of pregnane-3α,21-diol-11,20-dione diacetate (I) and Compound 5 did not form an insoluble digitonide, it was tentatively assigned the structure allopregnane-3α,11β,21-triol-20-one (II).



Since no reference standard was available, it was necessary to degrade the compound to a known alloetianic acid. A parallel degradation was carried out with allopregnane-3β,11β,21-triol-20-one (Reichstein's Substance R) (III) (14, 15). The two methyl esters (IV and V) had distinctly different absorption in the 1350 to 750 cm.$^{-1}$ region, but both had similar strong bands in the carbonyl region characteristic of carbomethoxy groups. The methyl alloetianate (V) derived from Substance R had its maximum at 1738 cm.$^{-1}$, while that from Compound 5 (IV) showed maximal absorption at 1736 cm.$^{-1}$.

Chromic acid oxidation of the two methyl esters yielded substances which had identical infra-red spectra throughout the range usually measured. The degradation path from III makes it apparent that VI must be methyl 3,11-diketoalloetianate.

Further support for this structure was obtained by examination of the

infra-red spectra of the acetates VII and VIII. Acetate VII had the complex acetate absorption (1256, 1237 cm.$^{-1}$) characteristic of the axial conformation (16) (3α,5α or 3β,5β), while VIII, which has the equatorial conformation (3β,5α or 3α,5β), had a single band at 1240 cm.$^{-1}$. Since Compound 5 does not form an insoluble digitonide and has been degraded to an alloetianic acid, there can be little doubt that it is allopregnane-3α,11β,21-triol-20-one (II).[2]

Characterization of Zone 6—The eluate from Zone 6 was further purified by passage through a column of silica gel. Examination of the eluate in the infra-red revealed strong hydroxyl absorption and a band at 1708 cm.$^{-1}$ (20-ketone). Acetylation yielded a substance which still had hydroxyl absorption and, in addition, bands at 1755 and 1734 cm.$^{-1}$ characteristic of a 20-keto-21-acetoxy structure. Chromic acid oxidation of the acetylated material yielded a substance the infra-red absorption of which was identical in every respect with that of the authentic sample of I and the diacetate of Compound 4. Compound 6 is therefore pregnane-3α,11β,21-triol-20-one and its diacetate is IX.

Non-Ketonic Fraction—The total non-ketonic portion of the formaldehydogenic lipides was subjected to a 150 transfer counter-current distribution, and selected tubes were analyzed for formaldehydogenic lipides and acylable lipides. The principal acylable lipide was found at transfer numbers 130 to 110. A small amount of formaldehydogenic lipide was also present in this region. The contents of these tubes were combined, oxidized with periodic acid, and the neutral oxidation products freed from carbonyl compounds with the aid of Girard's Reagent T. After acetylation, the infra-red spectrum of the non-ketonic fraction was indistinguishable from that of an authentic specimen of pregnane-3α,20α-diol-11-one diacetate.

DISCUSSION

Counter-current distribution and paper chromatography were used as complementary techniques for the resolution of the steroid mixture isolated from urine after β-glucuronidase hydrolysis. While the number of transfers applied was insufficient to separate the individual components, the character of the curve provided evidence for the presence of more than one component in the major band. In the case of the non-ketonic fraction, the application of two analytical parameters revealed the presence of a formaldehydogenic substance which contaminated the major alcoholic component.

[2] Direct comparison of the infra-red spectrum of Compound 5 diacetate with that of a sample of allopregnane-3α,11β,21-triol-20-one 3,21-diacetate prepared by partial synthesis completely established their identity. The synthetic material was generously sent us by Dr. F. C. Dohan.

Subsequently, paper chromatography was used to resolve the major components of the ketonic fraction, three of which were identified, two by direct comparison with authentic specimens and the third by degradation to a known compound. Since the quantity of material was insufficient for characterization of intermediates and the final product by classical procedures, the structural conclusions were based almost entirely upon spectral evidence. However, the chemical evidence and spectral evidence are wholly consistent with one another.

It is noteworthy that the principal α-ketol isolated from the urine of this patient during the administration of corticosterone should belong to the $3\alpha,5\alpha$ series (Table II). This is in contrast to the metabolites of progesterone, deoxycorticosterone, cortisone, and hydrocortisone whose major metabolites belong to the $3\alpha,5\beta$ series (17). Whether this departure from

TABLE II

Urinary Metabolites of Administered Corticosterone

Source	Compound	Weight*	Yield†
		mg.	*per cent*
Zone 4	Pregnane-3α,21-diol-11,20-dione	2.4	1.0
" 5	Allopregnane-3α,11β,21-triol-20-one	14.0	5.8
" 6	Pregnane-3α,11β,21-triol-20-one	5.9	2.5
"Non-ketonic"	Pregnane-3α,20α-diol-11-one	7.9	3.3

* Based on assay for formaldehydogenic lipides.

† Based on administered corticosterone.

the usual pattern is structurally determined or is a metabolic characteristic of the particular patient or of his disease cannot be ascertained from this experiment. It should be mentioned that for the compounds thus far isolated the ratio of 5β to 5α steroids is 1.1.

We wish to thank Dr. Walter Bauer and Dr. William S. Clark for making available the urine specimens, Dr. L. H. Sarett for his gift of pregnane-3α,21-diol-11,20-dione diacetate, and Dr. A. Zaffaroni for his gift of allopregnane-3β,11β,21-triol-20-one (Reichstein's Substance R). Our thanks are also due Miss Marjorie J. Springer and Miss Faiza A. Fawaz for measuring the infra-red spectra.

SUMMARY

Allopregnane-3α,11β,21-triol-20-one, pregnane-3α,11β,21-triol-11-one, pregnane-3α,21-diol-11,20-dione, and pregnane-3α,20α-diol-11-one have been isolated from the urine of a patient treated with corticosterone.

XUM

BIBLIOGRAPHY

1. Mason, H. L., *J. Biol. Chem.*, **172,** 783 (1948).
2. Lieberman, S., Fukushima, D. K., and Dobriner, K., *Federation Proc.*, **7,** 168 (1948).
3. Gómez Mont, F., and Berliner, D., *Gaceta med. Mexico*, **83,** 413 (1953).
4. Cohen, S. L., *J. Biol. Chem.*, **192,** 147 (1951).
5. Hollander, V. P., DiMauro, S., and Pearson, O. H., *Endocrinology*, **49,** 617 (1951).
6. Girard, A., and Sandulesco, G., *Helv. chim. acta*, **19,** 1095 (1936).
7. Craig, L. C., and Craig, D., in Weissberger, A., Techniques of organic chemistry, New York, **3,** 171 (1950).
8. Engel, L. L., Alexander, J., Carter, P., Elliott, J., and Webster, M., *Anal. Chem.*, **26,** 639 (1954).
9. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.*, **188,** 763 (1951).
10. Zaffaroni, A., and Burton, R. B., *J. Biol. Chem.*, **193,** 749 (1951).
11. McKay, A. F., *J. Am. Chem. Soc.*, **70,** 1974 (1948).
12. Baggett, B., Engel, L. L., and Fielding, L. L., *J. Biol. Chem.*, **213,** 87 (1955).
13. Jones, R. N., and Dobriner, K., *Vitamins and Hormones*, **7,** 293 (1949).
14. Reichstein, T., and von Euw, J., *Helv. chim. acta*, **21,** 1197 (1938).
15. Reichstein, T., *Helv. chim. acta*, **21,** 1490 (1938).
16. Jones, R. N., Humphries, P., Herling, F., and Dobriner, K., *J. Am. Chem. Soc.*, **73,** 3215 (1951).
17. Dorfman, R. I., *J. Clin. Endocrinol. and Metabolism*, **14,** 318 (1954).

A SENSITIVE METHOD FOR THE DETERMINATION OF DEOXYRIBONUCLEIC ACID IN TISSUES AND MICROORGANISMS

By JUNIUS M. WEBB and HILTON B. LEVY

(From the Laboratory of Infectious Diseases, National Microbiological Institute, National Institutes of Health, Bethesda, Maryland)

(Received for publication, August 20, 1954)

The increasing interest in deoxyribonucleic acid (DNA) and its function in cellular physiology has made desirable specific and more sensitive methods for its determination in biological material. Perhaps the most widely used method is that of Dische (1) in which diphenylamine is used to develop a blue color with the carbohydrate moiety from the purine nucleotides of the nucleic acid. The method has been criticized on the basis of protein (2) and carbohydrate (3) interference.

Among less frequently employed methods are the cysteine method (4), based on an earlier observation of Dische that a pink color is formed when DNA is heated with cysteine and sulfuric acid, and the tryptophan method (3), in which both the purine-bound and the pyrimidine-bound carbohydrate are estimated by a color reaction with tryptophan in the presence of perchloric acid.

Recently, a micromethod which employs indole as the reagent has been described (5). It is claimed to be about 10 times as sensitive as the diphenylamine test. However, no data are presented concerning its value when used with tissue or microorganisms.

The present paper reports a color test in which *p*-nitrophenylhydrazine is shown to measure quantitatively the deoxyribose of DNA. The specific extinction coefficient of the colored product found with the proposed reagent is about 5 times that of the colored product with diphenylamine when optical densities are taken at their respective wave-lengths of maximal absorption. Further, the color absorption of the blank in the method to be described is a small, reproducible portion of the total absorption. The larger amount of color in the diphenylamine blank makes the determination of small amounts of DNA with this reagent unreliable. Experiments performed with a variety of proteins and carbohydrates show the proposed method to be as specific as or more specific than the diphenylamine test.

EXPERIMENTAL

Reagents—

1. *p*-Nitrophenylhydrazine, Eastman Kodak Company, Rochester, New

York. The material was found to be suitable without recrystallization. 5 or 10 ml. of 0.5 per cent solution in ethanol were prepared fresh daily.

2. *n*-Butyl acetate, Baker's purified.

3. Trichloroacetic acid (TCA), Merck reagent.

4. Deoxyribonucleic acid. The DNA was prepared from calf thymus by the method of Hammarsten (6) and gave an analysis of N 12.63 per cent, P 6.95 per cent, N:P ratio 1.82.

Materials Analyzed for DNA—

1. Rat tissues. The livers, lungs, kidneys, and spleens from young Sprague-Dawley rats were removed immediately after sacrificing the animals and were frozen at once in dry ice.

2. Yeast. Bakers' yeast (Fleischmann), dried *in vacuo*, was used.

3. Bacteria. *Proteus vulgaris* and *Escherichia coli*, from 17 hour cultures grown at 37° on horse meat infusion agar, were harvested in 50 ml. of 0.85 per cent saline. The suspensions were treated with 1 ml. of 37 per cent formaldehyde and the residues recovered by centrifugation for 10 minutes at 20,000 × *g* in a Servall SS-1 centrifuge. The residues were washed twice with distilled water, recovered by centrifugation, and dried *in vacuo*.

The preparation of biological material for analysis was in general accomplished by the method of Schneider (7), followed by dehydration with ether and drying *in vacuo* to give a dry powder from which convenient, reproducible aliquots could be taken. Recoveries were made as carefully as possible in order that the amount of dried powder used could be related to the weight of fresh animal tissue or dried microorganisms employed for the original homogenate. Homogenization was carried out in a Waring blendor. In certain experiments, to be detailed later, extracted material without alcohol-ether treatment or conversion to powder was used.

Analytical Procedure

Hydrolysis—The material to be analyzed was suspended in 3 ml. of 5 per cent TCA and the suspension heated for 30 minutes in a boiling water bath. 1 to 20 mg. of the dried powder, containing 5 to 150 γ of DNA, was taken per ml. of TCA. This amount of dried powder is equivalent to approximately 6 to 120 mg. of fresh tissue. It was found convenient to carry out the hydrolysis in a 15 ml. conical centrifuge tube, in the mouth of which an inverted sealed ampul bulb was placed to minimize loss by evaporation. With this 3 ml. volume, loss by evaporation did not exceed 0.1 ml. during the 30 minute hydrolysis period. After hydrolysis and cooling in cold water, a volume of 5 per cent TCA equal to the original volume was added. The tube contents were mixed and centrifuged.

A stock solution of standard hydrolyzed DNA was prepared as follows: 25 mg. of DNA and 20 ml. of 5 per cent TCA were placed in a 25 ml.

volumetric flask, which was covered with a sealed ampul bulb and heated for 30 minutes in a boiling water bath. After hydrolysis and cooling, the volume was brought to 25 ml. with 5 per cent TCA. Standards of lower concentration were prepared by appropriate dilution of this hydrolyzed stock solution with 5 per cent TCA.

Color Development—Duplicate 2 ml. aliquots of the hydrolyzed material to be assayed, containing 10 to 300 γ of DNA, were transferred to 15 ml. conical centrifuge tubes, and 2 ml. of a standard solution containing 100 γ of hydrolyzed DNA were transferred to a similar tube. Into each were pipetted 2 ml. of 5 per cent TCA, followed by 0.2 ml. of *p*-nitrophenylhydrazine reagent. A blank, prepared simultaneously, consisted of 4 ml. of 5 per cent TCA to which was added 0.2 ml. of the reagent.

The tubes were then heated for 20 minutes in a boiling water bath, again with a sealed ampul bulb for a condenser. After cooling the tubes in cold water, about 10 ml. of *n*-butyl acetate were added to each tube. The tubes were stoppered with clean rubber stoppers and shaken for 5 minutes. The stoppers were then removed, the tubes centrifuged to complete the separation of the organic from the aqueous layers, and the greater portion of the organic layers of each tube was decanted and discarded. 3 ml. of the aqueous layer of each tube, taken by dipping a 3 ml. volumetric pipette beneath the remaining organic phase, were then transferred to a 5 ml. volumetric flask. 1 ml. of 2 N NaOH was added to develop the color. The solution was then diluted to volume with water and mixed.

The colored solution was quickly transferred to a Klett tube and the optical density measured immediately against a distilled water blank, contained in a matched tube, by means of a Klett-Summerson photometer equipped with a 540 mμ filter. For maximal sensitivity the colored solution should be read at its maximal absorption, 560 mμ, in a spectrophotometer.

Standardization and Validity of Method

Optimal Conditions for Hydrolysis—Schneider (7), employing the diphenylamine reaction, determined that hydrolysis at 90° for 15 minutes with 5 per cent TCA was optimal for splitting nucleoproteins. However, with the *p*-nitrophenylhydrazine reagent, it has been found that slightly greater color intensities were obtained with 30 minute hydrolysates. No further increase in color was observed when a 45 minute hydrolysate was used. Based on these observations, a 30 minute hydrolysis period was adopted.

Effect of Acid Concentration and Heating Time on Reaction between p-Nitrophenylhydrazine and Carbohydrate from Hydrolyzed DNA—The reaction with *p*-nitrophenylhydrazine was apparently little affected by the concen-

tration of acid between 1 and 10 per cent TCA. However, a final acid concentration of from 3 to 5 per cent TCA was necessary to effect quantitative extraction, with butyl acetate, of excess reagent and other compounds formed. The addition of fresh TCA after hydrolysis was found desirable, since part of the acid was lost by distillation or disappeared in other ways during the heating period. The additional amount of TCA also led to the precipitation of any protein that might still be in solution. Although 10 to 15 minutes appeared to be sufficient, the 20 minute heating period for the formation of the chromogenic compound was selected to allow ample time for the reaction to go to completion.

Color Stability—Maximal intensity of color developed almost immediately upon addition of the NaOH to the acid extract, after which the color started to fade. The rate of fading decreased after the first 5 minutes, during which time it was approximately 2 per cent a minute. Since all samples were read at about the same time (within a minute after color development), the error from fading was small. This fact was borne out by the consistent reproducibility of optical densities obtained on aliquots of the same solution.

The concentration and amount of NaOH used were not critical so long as enough was present to raise the pH to 11 or above. Above this pH, the rate of fading and intensity of color were not affected by more alkali.

The amount of reagent prescribed was found ample to react with more than 1 mg. of DNA. Larger amounts of reagent had no effect on the final color intensity so long as sufficient butyl acetate was used to extract the excess.

Standardization of Test—In order to show that the amount of colored product formed with *p*-nitrophenylhydrazine is directly proportional to the amount of DNA present, the color development procedure was applied to various dilutions of the hydrolyzed DNA standard solution. The resulting direct proportionality is shown in Fig. 1.

If there is no factor interfering as a result of TCA hydrolysis, the curve representing results obtained from dilutions of a sample already hydrolyzed and the curve obtained from individually hydrolyzed DNA samples should coincide over the same range of concentrations. Accordingly, five dilutions of an aqueous homogenate of known DNA content were hydrolyzed in 5 per cent TCA for 30 minutes, and the DNA was determined in each in duplicate. The optical densities were as expected from the curve of Fig. 1 and when plotted gave a superimposable straight line.

Spectral Characteristics—Spectral transmittance curves[1] presented in Fig. 2 show the absorption characteristics of the colored product resulting from

[1] The authors are indebted to Mr. Charles W. Hench of Beckman Instruments, Inc., Washington, D. C., for these spectrometric data.

the *p*-nitrophenylhydrazine reaction. For comparison, the curve for the colored product resulting from the diphenylamine reaction is also given. These curves represent solutions of the same concentration of DNA read against their respective blanks and illustrate the difference in the quantitative sensitivity of the two reactions. The extinction coefficients at their respective maxima (560 mμ for the *p*-nitrophenylhydrazine product and 600 mμ for the diphenylamine product) show the color of the former

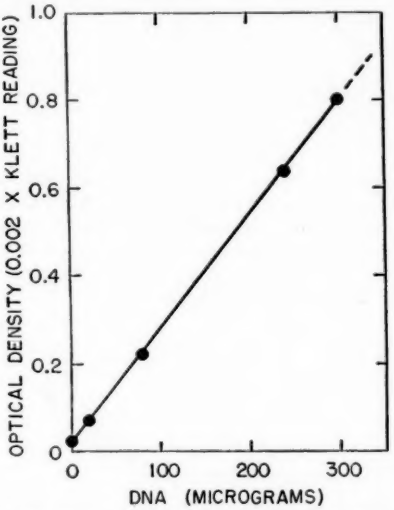


FIG. 1. Standardization curve, showing the relationship between optical density and amount of DNA, as obtained by the *p*-nitrophenylhydrazine method. The amounts of DNA represent the initial amounts of hydrolyzed DNA taken. The final aliquots used for color development contained the equivalent of 75 per cent of these initial amounts, as described in the assay procedure. Each point represents the mean of eight determinations. The readings were made against distilled water with matched 12 mm. tubes in the Klett photometer equipped with a 540 mμ filter.

at its maximum to be about 5 times as intense as the color of the latter at its maximum.

Relationship between Amounts of Tissue Powder and Amounts of DNA Found—Fig. 3 illustrates the results of a typical experiment in which the method was applied to increasing amounts of liver powder. It will be seen that there is a direct proportionality between the optical density readings and the amount of powder used.

Recovery of DNA Added to Tissue Powder—Known amounts of DNA were added to five samples of liver powder. To each 6.97 mg. portion of powder, 0, 12, 24, 48, 72, and 96 γ of DNA were added. After hydrolysis,

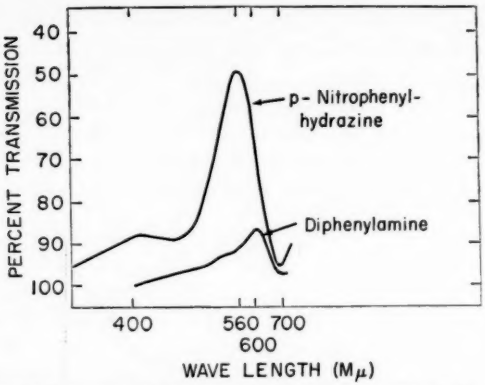


FIG. 2. Absorption spectra of the products of the *p*-nitrophenylhydrazine reaction and of the diphenylamine reaction with hydrolyzed DNA. The initial concentrations of hydrolyzed DNA were such that the final colored solution in each case was equivalent to 15 γ of DNA per ml. The curves were taken from a Beckman model DK recording spectrophotometer. Matched 10 mm. silica cells were used, and the solutions were read against their respective blanks.

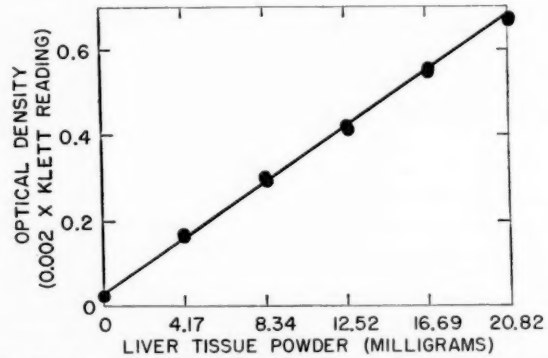


FIG. 3. Application of the *p*-nitrophenylhydrazine method to increasing amounts of liver tissue powder. Each amount of tissue powder shown represents an aliquot (one-third) of the amount weighed for hydrolysis. Duplicate determinations were made on each hydrolysate. As in Fig. 1, the final aliquots used for color development contained the equivalent of 75 per cent of the hydrolyzed DNA that was in the weight of tissue powder shown. The optical density readings were made as described in Fig. 1.

recovery of DNA by the *p*-nitrophenylhydrazine method ranged from 93 to 101 per cent of the amount added.

Effect of Alcohol-Ether Extraction in Tissue Preparation—Generally, in the preparation of tissues for DNA analysis, the tissue is extracted several

times with alcohol and ether after the acid-soluble components of the tissue have been extracted with cold TCA. Schneider (7), employing the diphenylamine method, reported little difference between tissues that were acid-washed and those which were also extracted in alcohol and ether.

Experiments were performed to investigate the necessity of the alcohol-ether extraction when *p*-nitrophenylhydrazine was used as the colorimetric reagent. Table I summarizes data obtained with rat liver and spleen.

TABLE I

Comparison of Results on Fresh Rat Tissues Extracted by Different Procedures

Tissue	Extraction procedure	Analysis No.	DNA, mg. per 100 gm. fresh tissue
Liver	10% TCA	1	199
			192
		2	188
			187
"	10% " and alcohol-ether	1	200
			196
		2	204
			190
Spleen	10% "	1	1480
			1445
		2	1435
			1420
"	10% " and alcohol-ether	1	1355
			1330
		2	1375
			1400

Since the hydrolysis was on the extracted tissue (without conversion to powder), the amount of DNA per gm. of fresh tissue can be calculated directly. It may be seen that there is no significant difference between the results obtained with tissues extracted with alcohol-ether and those with tissues not so extracted.

Specificity of p-Nitrophenylhydrazine Reagent—That the *p*-nitrophenylhydrazine reagent reacted with the carbohydrate moiety of DNA was shown by applying the method to deoxyribose anilide.[2] The resulting color had the same spectral maximum (560 mμ) as the color resulting from DNA. Further, deoxyribose anilide and DNA, estimated to contain the same amount of deoxyribose, gave approximately the same amount of color.

[2] Deoxyribose anilide may be obtained from the California Foundation for Biochemical Research, Los Angeles, California.

Little or no color developed under the conditions of the test with ribose, hydrolyzed RNA, or hydrolyzed ribosides when their final volumes, made

TABLE II

Analyses of Tissues and Microorganisms for DNA by p-Nitrophenylhydrazine Method and Diphenylamine Method*

Original material	Analysis No.	DNA, γ per mg. extracted powder		Conversion factor, mg. extracted powder per mg. original material	Average DNA, mg. per 100 gm. fresh tissue		Average DNA, mg. per gm. dry material	
		p-Nitrophenylhydrazine	Diphenylamine		*p*-Nitrophenylhydrazine	Diphenylamine	*p*-Nitrophenylhydrazine	Diphenylamine
Rat lung	1	48.9	56.2					
		52.2	59.1	0.122	598	653		
	2	45.8	50.7					
		49.2	48.3					
" liver	1	13.9	15.8					
		14.4	15.9	0.172	236	277		
	2	13.2	17.0					
		13.3	15.8					
" kidney	1	25.4	24.9					
		26.3	27.4	0.137	339	370		
	2	23.8	28.6					
		23.5	27.2					
Yeast	1	4.5	5.4					
		4.1	5.4	0.732			3.2	3.7
	2	4.2	5.0					
		4.7	4.4					
E. coli	1	62.8	60.9					
		60.3	60.7	0.692			41.4	42.0
	2	57.8	59.2					
		58.2	61.9					
P. vulgaris	1	48.4	49.2					
		47.9	52.2	0.767			38.5	37.0
	2	51.0	47.0					
		53.7	44.7					

* The diphenylamine assays were performed on separate hydrolysates of the extracted powders after the 15 minute hydrolysis in 5 per cent TCA as recommended by Schneider (7). Sample weights were taken so that the concentration of hydrolyzed DNA was the same as that in the hydrolysates used for the *p*-nitrophenylhydrazine assays.

alkaline after the butyl acetate extraction, contained the equivalent of 1 mg. of ribose. The same was true of the following carbohydrate materials less likely to be present: maltose, fructose, glucose, saccharose, methyl glucoside, glycogen (hydrolyzed), xylose, arabinose, and inulin (hydrolyzed).

In another experiment the proteins casein, egg albumin, edestin, and zein were hydrolyzed and treated with the reagent. When the final alkaline solutions of each represented 1 mg. of protein, the optical densities also were negligible as compared to the TCA blank.

Application and Reproducibility of Method

Analyses of Tissues and Microorganisms for DNA Content by p-Nitrophenylhydrazine Method and Diphenylamine Method—In order to compare the two methods, the analyses summarized in Table II were performed. It can be seen that the ranges of results by the two methods generally overlap. However, on the average, the *p*-nitrophenylhydrazine results are slightly lower than the diphenylamine values. The values found by both methods for lung, kidney, liver, *E. coli*, and yeast, when converted to mg. of phosphorus per 100 gm. of tissue, agree well with those given for the same materials by Davidson (8).

Reproducibility and Precision—The reproducibility of the *p*-nitrophenylhydrazine method as applied to the analyses of biological materials is exemplified by the data in Table II. With the six different DNA materials, on which two analyses in duplicate are reported, the per cent deviation of each determination from its respective mean of four such determinations can be calculated. By the *p*-nitrophenylhydrazine method, the average of all twenty-four of the per cent deviations was approximately ±4.0 and the maximal single per cent deviation was about 7.5. By the diphenylamine method, the average of all twenty-four of the per cent deviations was about the same, ±4.6, whereas the maximal single per cent deviation was approximately 13.

To determine the precision of colorimetry, amounts of four standard solutions representing 20, 80, 240, and 300 γ of hydrolyzed DNA were subjected to the colorimetric procedure eight times. When, in each case, the standard deviations were expressed as a per cent of their respective means, the values found were 2.4, 1.9, 2.3, and 2.6. These results indicate a satisfactory degree of precision over the range from 20 to 300 γ of DNA.

DISCUSSION

Inasmuch as the product of the reaction of *p*-nitrophenylhydrazine with hydrolyzed DNA has not been isolated, it can only be postulated that the deoxypentose resulting from the hydrolysis forms a hydrazone when heated with the phenylhydrazine derivative in the presence of TCA. Other carbohydrates present, which might be capable of reacting with *p*-nitrophenylhydrazine, either do not form products which are colored in alkaline medium or are extracted along with excess reagent by butyl

acetate when in TCA solution. To the best of the authors' knowledge, this reaction is a new approach to DNA analysis.

As has been pointed out, DNA values on biological materials found by the proposed method are, on the average, lower than the values by the diphenylamine method. These lower results may be explained by assuming that *p*-nitrophenylhydrazine does not give color with protein or protein breakdown products resulting from TCA hydrolysis. Such an explanation would be in line with the observations of others (9, 2) that, in the presence of proteins or protein degradation products, the diphenylamine method gives high DNA values. That protein can still remain in the TCA hydrolysate after heating has been shown by others (10) and observed in this laboratory.

Some investigators (1, 11), using the diphenylamine method, employ a complicating additional control to correct for non-specific color resulting from the action of sulfuric acid on the tissue or biological material extracts. With *p*-nitrophenylhydrazine, as shown in Fig. 3, non-specific color did not develop from the extract of liver tissue powder in the presence of the milder TCA. In view of the generally lower results, with the *p*-nitrophenylhydrazine method, one might assume that the same applies to other materials.

The preliminary experiments, presented herein, indicate that, in the preparation of liver and spleen for analysis, the extraction with alcohol and ether is not necessary. Perhaps it can also be omitted in the preparation of other biological materials.

The specificity and sensitivity of the *p*-nitrophenylhydrazine reagent for deoxyribose, coupled with the butyl acetate extraction procedure, suggest the possibility for its use in chromatography. Although there has been no experimentation in this direction, the identification or isolation of minute amounts of deoxyribose would seem possible by such methods.

SUMMARY

Deoxyribonucleic acid hydrolyzed in trichloroacetic acid reacted quantitatively with *p*-nitrophenylhydrazine. When the product was separated from interfering substances, it was determinable in alkaline solution colorimetrically. The developed color followed Beer's law over the range of 10 to 300 γ of DNA. *p*-Nitrophenylhydrazine was specific for deoxyribose under the conditions of the test and showed greater sensitivity than diphenylamine, the reagent usually employed for DNA assay. The results were reproducible over the assay range, and good DNA recoveries were obtained with minimal interference. The DNA values found for

several rat tissues and microorganisms agreed well with the ranges of values given in the literature.

BIBLIOGRAPHY

1. Dische, Z., *Mikrochemie*, **8**, 4 (1930).
2. von Euler, H., and Hahn, L., *Svensk Kem. Tidskr.*, **58**, 251 (1946).
3. Cohen, S. S., *J. Biol. Chem.*, **156**, 691 (1944).
4. Stumpf, P. K., *J. Biol. Chem.*, **169**, 367 (1947).
5. Ceriotti, G., *J. Biol. Chem.*, **198**, 297 (1952).
6. Hammarsten, E., *Biochem. Z.*, **144**, 383 (1924).
7. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
8. Davidson, J. N., The biochemistry of the nucleic acids, London, 65, 131 (1950).
9. Dounce, A. L., *Exp. Cell Res.*, suppl. 2, 103 (1952).
10. Solodkowska, W., and von Euler, H., *Svensk Kem. Tidskr.*, **58**, 116 (1946).
11. Seibert, F. B., *J. Biol. Chem.*, **133**, 593 (1940).

CONVERSION OF INORGANIC I^{131} TO ORGANIC I^{131} BY CELL-FREE PREPARATIONS OF THYROID TISSUE*

By ALVIN TAUROG, G. D. POTTER, AND I. L. CHAIKOFF

(From the Department of Physiology of the University of California School of Medicine, Berkeley, California)

(Received for publication, September 13, 1954)

The synthesis of organic iodine compounds by surviving slices of thyroid tissue has been described in previous communications from this laboratory (1, 2). More recently, formation of organic iodine by thyroid homogenates and by isolated thyroid mitochondria and nuclei was reported by Weiss (3). According to this worker, the addition of both copper and tyrosine is necessary for synthesis of organic iodine by these systems. Weiss suggested that a copper-containing enzyme may be involved in the iodination of tyrosine by thyroid tissue.

Fawcett and Kirkwood (4) questioned Weiss' interpretation of the rôle of copper in the synthesis of organic iodine by thyroid homogenates reenforced with tyrosine. These workers concluded that the copper did not act enzymatically, but simply oxidized the added iodide-I^{131} to I_2^{131}, the latter then iodinating the added tyrosine. They postulated the presence, in thyroid tissue, of an enzyme "tyrosine iodinase" which catalyzes the iodination of added tyrosine by iodine (4, 5). Fawcett and Kirkwood employed filter paper chromatography rather than the less specific butanol extraction procedure used by Weiss to separate the iodine compounds of the thyroid. They found that monoiodotyrosine was the only organic iodine compound formed under the conditions of their experiments, and they concluded, therefore, that the enzyme catalyzes only a monoiodination of tyrosine. Their studies emphasized iodination of *added* tyrosine. In the absence of either added copper or tyrosine, the thyroid homogenates formed negligible amounts of organic iodine.

The results obtained with thyroid homogenates in our laboratory differ in certain essential respects from those reported by previous workers. We find that neither copper nor tyrosine need be added in order to effect organic iodine formation in thyroid homogenates or thyroid particulate fractions. These cell-free preparations, not reenforced with any additional substrates, convert appreciable quantities of added inorganic I^{131} to monoiodotyrosine. This monoiodotyrosine, however, unlike that formed in the experiments of Fawcett and Kirkwood, is not free, but is released only upon hydrolysis of thyroid protein. A second I^{131}-containing compound,

* Aided by a grant from the United States Public Health Service.

XUM

not bound to protein and not yet identified, is also formed under the conditions of our experiments. In agreement with Fawcett and Kirkwood, we also find that very little diiodotyrosine-I^{131} and practically no thyroxine-I^{131} are formed after disruption of the thyroid cells.

EXPERIMENTAL

Most of the experiments reported here were carried out with sheep thyroid glands. These were obtained at the slaughter-house, from freshly slaughtered sheep, and carried back to the laboratory packed in ice. The fractionation procedures were begun within an hour after removal of the thyroid glands from the animals. At all stages of the fractionation the tissue was kept at a temperature of 4° or less.

Preparation of Homogenates—Thyroid tissue contains much more connective tissue than does liver or kidney, and for this reason it is not so readily homogenized. In our early experiments, the glands were first frozen in liquid nitrogen and powdered in a stainless steel mill that had been cooled with solid carbon dioxide. The frozen powder was then homogenized in a Potter-Elvehjem type homogenizer, with either 0.88 M sucrose or Krebs-Ringer bicarbonate solution. Although this procedure yielded active particulate fractions, it was found later that slightly more active preparations could be obtained if the tissue were not frozen previously. The procedure finally adopted was as follows: The thyroid glands were first sliced with a razor blade, and the slices were collected in ice-cold Krebs-Ringer bicarbonate solution (6). Portions of the slices, weighing 1.5 gm., were minced with scissors, transferred to a chilled glass homogenizer tube (Arthur H. Thomas catalog No. 4288-B), and homogenized with 9 to 10 cc. of ice-cold Krebs-Ringer bicarbonate buffer. Since the thyroid tissue would not grind well when the original, tight fitting Teflon plunger was used, the homogenization was carried out with a slightly loose plunger. (This was obtained by reducing the diameter of the standard plunger very slightly on a lathe.) The homogenizer tube was surrounded by a beaker of ice during the grinding procedure. After this preliminary homogenization, the tissue was homogenized once again (this time with a tight fitting plunger) to insure complete disruption of cells. The homogenate was filtered through fine mesh nylon cloth into a chilled flask. Several such homogenates were pooled before isolation of the particulate fractions.

Isolation of Thyroid Nuclei—The thyroid homogenate was poured into chilled, 50 cc. centrifuge tubes and centrifuged at 600 × g for 10 minutes at 0°. The supernatant fluids were drawn off with gentle suction, for isolation of the mitochondrial fraction (see below), and the residues, containing the nuclei, were resuspended in 10 cc. of Krebs-Ringer bicarbonate solution and centrifuged once again at 600 × g for 10 minutes at 0°. The supernatant fluids were discarded, and the washing of the residue

with 10 cc. of bicarbonate buffer was repeated. After centrifugation, the residue was suspended in 3 cc. of Krebs-Ringer bicarbonate solution for each 1.5 gm. of original tissue and homogenized lightly for a few moments to obtain a uniform suspension. Two different nuclear preparations were examined under the phase microscope. The nuclei were somewhat distorted in shape, but the preparations seemed relatively free of whole thyroid cells and mitochondria. Red blood cells were the major contaminant.

Isolation of Thyroid Mitochondria and Microsomes—The original supernatant fluids from the nuclear fraction (not including washings) were centrifuged for 30 minutes in a model L Spinco ultracentrifuge at 30,000 r.p.m. (average centrifugal force, 78,000 $\times$ g). The fraction which sedimented under these conditions contained both the mitochondria and the microsomes of the original homogenate. A preliminary experiment, in which mitochondria were separated from microsomes by centrifugation at 25,000 $\times$ g, indicated that the activities of these two fractions were not greatly different. In all subsequent experiments, therefore, they were treated as one fraction. The material sedimented by the Spinco centrifugation was suspended in Krebs-Ringer bicarbonate solution (3 cc. for each 1.5 gm. of original tissue), and the mixture was lightly homogenized to provide a uniform suspension. For the sake of simplicity, this suspension is hereinafter referred to as the mitochondrial fraction, even though it also contained microsomes.

Incubation of Thyroid Fractions with I^{131}—3 cc. of whole thyroid homogenate or of mitochondrial or nuclear suspension (in Krebs-Ringer bicarbonate solution) were placed in 25 cc. Erlenmeyer flasks. The whole homogenate represented approximately 450 mg. of the original wet tissue, and the mitochondrial and nuclear suspensions represented approximately 1.5 gm. of the original wet tissue. Radioactive iodide (50 to 100 μc.) was added as 50 or 100 μl. of a purified solution containing approximately 0.2 γ of iodide. Inhibitors and other substances, when they were present, were added in 25 or 50 μl. of solution or as a weighed amount of solid. The air in the reaction flasks was replaced with 95 per cent O_2-5 per cent CO_2, and the flasks were shaken in a constant temperature bath at 37° for 2 hours. When anaerobic conditions were desired, the tissue homogenate and its particulate fractions were suspended in Krebs-Ringer bicarbonate buffer which had been flushed with 95 per cent N_2-5 per cent CO_2, and the air in the reaction flasks was replaced with the same gas mixture.

For tissue slice experiments, 250 to 300 mg. of thyroid slices, prepared by hand with a razor blade, were suspended in 3 cc. of Krebs-Ringer bicarbonate solution to which 50 or 100 μl. of I^{131} solution had been added.

Treatment of Samples after Incubation—As each incubation flask was removed from the constant temperature bath, it was cooled in an ice bath,

XUM

and 25 μl. of 0.08 M thiouracil solution were added to stop formation of organic iodine. The pH was adjusted to 8.0 to 8.5 with dilute NaOH, and 0.5 cc. of the reaction mixture was hydrolyzed, with 5 or 10 mg. of pancreatin (Merck), at 37.5° for 8 hours, under toluene. (Increasing the time of hydrolysis beyond 8 hours did not increase the yield of free iodinated amino acids.) 20 μl. of the hydrolysate were chromatographed on filter paper in a collidine-water-NH_3 system as previously described (7). A similar aliquot of the unhydrolyzed reaction mixture was also subjected to filter paper chromatography. In the tissue slice experiments, the slices and medium were homogenized together after the incubation, and 20 μl. of the homogenate were delivered for chromatography before and after enzymatic hydrolysis. It was possible to chromatograph tissue suspensions and their hydrolysates directly (*i.e.*, without previous extraction), provided only small aliquots (20 μl.) were delivered on the filter paper.

The presence of a small concentration of thiouracil (7×10^{-4} M) in the mixture to be chromatographed minimized the occurrence of artifacts, most likely by keeping the iodide in reduced form.

A quantitative estimate of the radioactivity in the various bands on the filter paper chromatograms was made with the aid of a strip counter. The latter was described in a previous communication (8).

Purification of Radioactive Iodine—The radioactive iodine which was added to the incubation flask was always distilled from the original solution provided by the Oak Ridge National Laboratory. The procedure was as follows: 1 cc. of solution was placed in a small distilling bulb (5 cc. capacity) equipped with a standard taper joint (10/30); to this were added 5 γ of iodide carrier, 50 μl. of 18 N H_2SO_4, and 25 μl. of 30 per cent H_2O_2. A boiling chip was added, and the bulb was connected to a small, water-cooled condenser. The tip of the condenser dipped below the level of the receiving solution which consisted of 1 cc. of 0.001 M Na_2SO_3 in a graduated, 15 ml. centrifuge tube. The distilling bulb was heated with a small Glas-Col mantle, and distillation of the I^{131} was usually complete within 5 minutes after the reaction mixture had begun to boil. 1 drop of 1 per cent NaOH was added to the receiving solution which was diluted with distilled water to the desired volume, usually 2 to 3 cc.

Iodine and Nitrogen Analyses—Iodine determinations were performed directly on the tissue preparations, by the method previously described (9). Nitrogen determinations were performed by the Kjeldahl method. All analyses were made in duplicate.

Results

Organic Iodine Formation by Thyroid Tissue Slices—Thyroid tissue slices rapidly concentrate iodide from the surrounding medium and convert

it to thyroprotein. This is illustrated in Fig. 1, *a*, which shows the nature of the I^{131} in sheep thyroid slices after incubation with inorganic I^{131} for 2 hours. Practically all of the I^{131} on the chromatogram is divided between the thyroglobulin-like protein at the origin and inorganic iodide. No *free* iodinated amino acids are visible on the radioautograph.

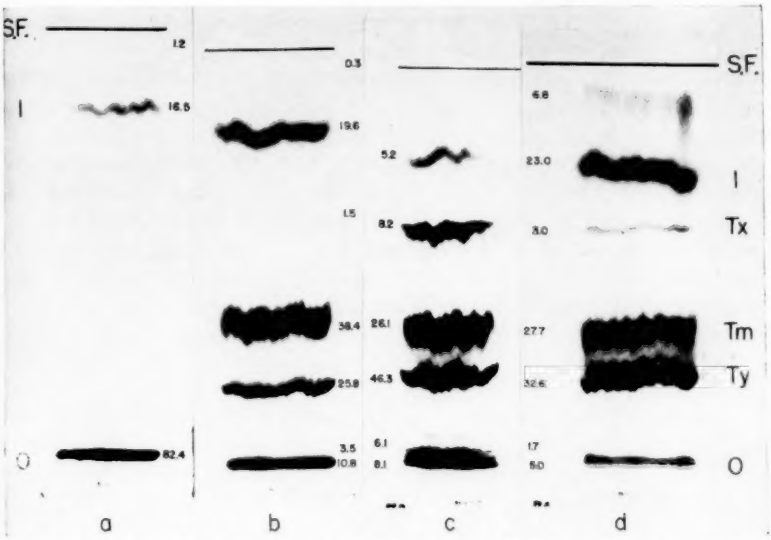


FIG. 1. Formation of organic I^{131} by thyroid tissue *in vitro* and *in vivo*. Radioautographs of chromatograms of (*a*) sheep thyroid tissue slices incubated with I^{131} for 2 hours, unhydrolyzed; (*b*) the same thyroid tissue slices as in (*a*) after hydrolysis with pancreatin; (*c*) thyroids of rats removed 4 hours after injection of I^{131} and hydrolyzed with pancreatin; (*d*) rat thyroid slices incubated with I^{131} for 2 hours and then hydrolyzed with pancreatin. The numbers indicate the percentage of the I^{131} on the chromatogram present in the various components. *O*, origin; *Tm*, monoiodotyrosine; *Ty*, diiodotyrosine; *Tx*, thyroxine; *I*, inorganic iodide; *S. F.*, solvent front. (Very weak areas of darkening on the original radioautographs do not appear in the photographic reproductions.)

The same thyroid sample is shown in Fig. 1, *b* after it had been hydrolyzed with pancreatin. The I^{131} in the thyroprotein had now been converted primarily to free mono- and diiodotyrosine. These two amino acids account for over 60 per cent of the I^{131} on the chromatogram. Thyroxine is formed only to a very small extent under these conditions. Even after 8 hours of incubation of sheep thyroid slices with iodide-I^{131}, formation of thyroxine-I^{131} was very low. The incompletely hydrolyzed protein remaining at the origin of the chromatogram contained only a negligible

amount of thyroxine; this was shown by eluting it with dilute alkali hydrolyzing with 2 N NaOH, and rechromatographing.

To demonstrate that pancreatin hydrolysis as used here is capable of releasing free thyroxine from thyroglobulin, the procedure was applied to thyroids removed from rats 4 hours after they had been injected with I^{131}. The glands were homogenized with slightly alkaline Krebs-Ringer bicarbonate (pH 8.3, 7×10^{-4} M thiouracil added), and the resulting homogenate was incubated with pancreatin (10 mg. per cc.) for 8 hours. The hydrolysate was chromatographed, and the radioautograph of the chromatogram is shown in Fig. 1, *c*. It is evident that the enzyme hydrolysis procedure is quite capable of releasing free thyroxine from thyroglobulin, thus lending confidence to the findings reported here, that sheep thyroid slices form only small amounts of thyroxine-I^{131}. The larger amounts reported in previous communications from this laboratory (1, 2) were based upon alkaline hydrolysis, followed by the relatively nonspecific butanol extraction procedure, and are undoubtedly incorrect. It is becoming increasingly clear that strongly alkaline hydrolysis may introduce serious errors into the analysis of mixtures of I^{131} compounds (10, 11). Much of the earlier work based on this procedure should be reexamined.

Rat thyroids were somewhat more active than were sheep thyroids in forming thyroxine *in vitro* (Fig. 1, *d*). This was so, even though the thyroids of rats were incubated as chunks of tissue (halves of each lobe), whereas the sheep glands were incubated as thin, uniform slices.

Organic Iodine Formation by Whole Thyroid Homogenate and Its Various Fractions—When thyroid tissue was homogenized, its ability to form organic I^{131} was reduced much below that of tissue slices. Fig. 2, *a* is the radioautograph of the chromatogram obtained after incubating 3 cc. of whole thyroid homogenate (approximately 450 mg. of tissue, 3.40 mg. of N per cc.) with I^{131} for 2 hours. The homogenate was hydrolyzed with pancreatin before it was chromatographed on filter paper. The band containing approximately 5 per cent of the total activity is monoiodotyrosine. No diiodotyrosine or thyroxine bands are visible on the radioautograph. The band which moves almost with the solvent front has not been identified. It is especially prominent in the mitochondrial fraction (see below), but it is usually relatively weak in tissue slices or in the glands of intact rats. The significance of this component remains to be determined.

The mitochondrium-microsome fraction derived from the above homogenate showed considerably more activity per mg. of N than did the original homogenate. When 3 cc. of this fraction, containing 1.30 mg. of N per cc., were incubated with iodide-I^{131}, almost 20 per cent of added I^{131} was

converted to monoiodotyrosine (Fig. 2, *b*). Only a small amount of the added I^{131} was incorporated into diiodotyrosine and practically none into thyroxine. The unknown band which runs near the solvent front was most striking in the mitochondrial fraction, amounting to over 35 per cent of the total activity. The formation of monoiodotyrosine-I^{131} by several different sheep thyroid mitochondrial preparations is shown in Table I.

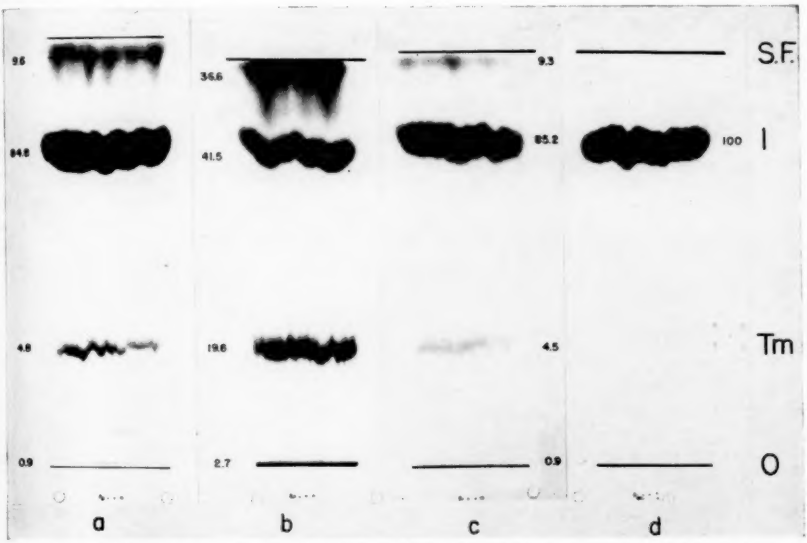


FIG. 2. Formation of organic I^{131} by whole thyroid homogenate and its various fractions incubated for 2 hours with iodide-I^{131} in Krebs-Ringer bicarbonate buffer. Radioautographs of chromatograms of material after hydrolysis with pancreatin. *a*, whole thyroid homogenate of sheep; *b*, mitochondrial fraction; *c*, nuclear fraction; *d*, soluble fraction. See Fig. 1 for the explanation of numbers and symbols and Table II, Homogenate Preparation 1, for nitrogen and iodine analyses of the various fractions.

The nuclear fraction (0.77 mg. of N per cc.) also revealed some activity. The radioautograph in this case (Fig. 2, *c*) was similar to that obtained with whole homogenate. Monoiodotyrosine accounted for 4.5 per cent of the total I^{131}, and the component near the solvent front was again quite prominent. No diiodotyrosine or thyroxine bands were visible. It does not seem likely to us that the activity of the nuclear fraction can be attributed to contamination by whole thyroid cells or mitochondria. However, since we cannot be certain that this is the case, the question of the formation of organic I^{131} by isolated thyroid nuclei must remain open.

The soluble fraction of the thyroid homogenate (2.74 mg. of N per cc.) showed no activity whatsoever in forming organic I^{131} from added inorganic I^{131} (Fig. 2, *d*).

Comparison of Radioautographs before and after Hydrolysis—The monoiodotyrosine-I^{131} which is formed by the subcellular particles of the thyroid and by the whole thyroid homogenate is part of the thyroid protein. It is released only upon hydrolysis of the protein, as illustrated in Fig. 3. Fig. 3, *a* shows the radioautograph of a chromatogram of a mitochondrial suspension incubated with I^{131} and delivered for chromatography *before* hydrolysis. The activity is distributed approximately equally among three different bands: (1) a thyroglobulin-like band at the origin; (2) an inorganic iodide band; and (3) an unidentified band near the solvent front. No free monoiodotyrosine band is visible on the radioautograph. Fig. 3,

TABLE I

Activity of Different Sheep Thyroid Mitochondrial Preparations in Forming Monoiodotyrosine-I^{131}

Homogenate preparation No.	Mitochondrial fraction		
	Mg. N per cc.	Per cent of I^{131} converted to monoiodotyrosine-I^{131}	Fig. illustrating activity of mitochondrial preparation
1	1.30	19.4	2, *b*
2	1.14	16.7	
3	1.27	27.2	3, *b*
4	1.54	27.6	4

b is the same sample after hydrolysis with pancreatin (10 mg. per cc.) for 8 hours. The thyroglobulin-like band at the origin has almost disappeared, and a strong monoiodotyrosine band containing 27 per cent of the total activity now appears on the radioautograph. There is a relative increase in the inorganic I^{131} fraction, indicating possible release of inorganic iodide during the enzyme digestion. It is of interest that the unidentified component at the solvent front, unlike the monoiodotyrosine, is present to approximately the same extent both before and after hydrolysis of the thyroid protein. Apparently this iodine compound is not an integral part of the thyroid protein.

In the case of the nuclear fraction also, comparison of the radioautographs of chromatograms prepared before and after hydrolysis reveals that monoiodotyrosine-I^{131} is formed as a component of the thyroglobulin-like protein (Fig. 3, *c* and *d*).

Time-Curve of Organic Formation by Thyroid Mitochondria; Effect of Added Iodide—The formation of monoiodotyrosine from added inorganic

I^{131} by thyroid mitochondria increases with time (Fig. 4). The increase is continuous at least up to 4 hours. After that, the activity levels out. It is interesting that formation of diiodotyrosine-I^{131} does not increase with increasing time of incubation. Even after 8 hours of incubation with I^{131}, the mitochondria formed only a trace of diiodotyrosine-I^{131}.

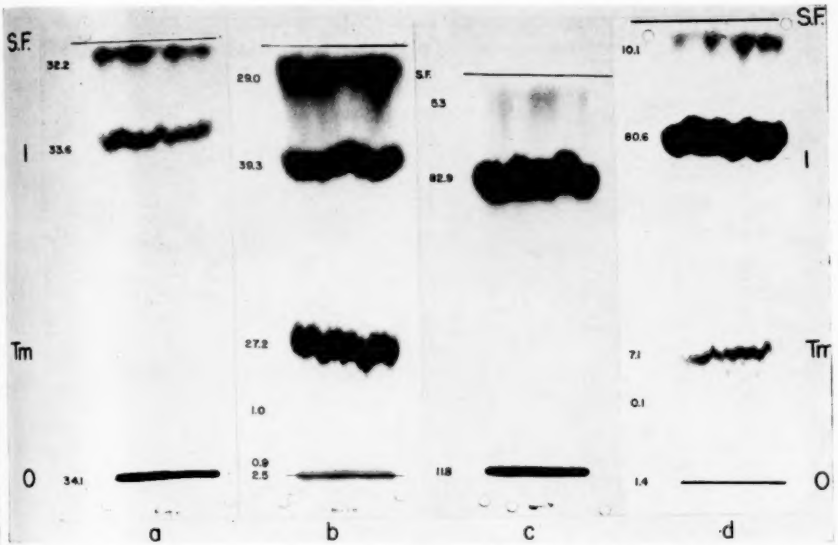


FIG. 3. Radioautographs of chromatograms of sheep thyroid mitochondrial and nuclear fractions incubated with I^{131} for 2 hours. *a*, mitochondrial fraction, unhydrolyzed; *b*, mitochondrial fraction, after hydrolysis with pancreatin; *c*, nuclear fraction, unhydrolyzed; *d*, nuclear fraction, after hydrolysis with pancreatin. See Fig. 1 for the explanation of numbers and symbols; for nitrogen and iodine analyses see Table II, Homogenate Preparation 3 for mitochondria and Homogenate Preparation 2 for nuclei.

It seemed that the lack of formation of diiodotyrosine might have been due to the presence of insufficient iodide in the surrounding medium. In most experiments, the iodide concentration was approximately 0.1 γ per cc. Even when this was raised to 1.7 γ per cc., however, there was still no effect on the formation of diiodotyrosine-I^{131}. Some other limiting factor, therefore, is involved in the failure of mitochondrial suspensions to form appreciable amounts of diiodotyrosine-I^{131} when incubated with iodide-I^{131}.

Formation of Organic I^{131} by Non-Thyroid Tissue Fractions—Mitochondrial and nuclear fractions were prepared from rat liver and rat kidney cortex and incubated with I^{131} exactly as described for thyroid tissue.

Radioautographs of chromatograms of such samples are presented in Fig. 5. It is evident that no organic I^{131} is formed by these tissue fractions under the conditions used here. Formation of organic iodine from inorganic iodide is not, therefore, a generalized property of all mitochondria or nuclei.

Effect of Various Inhibitors and Cofactors on Organic I^{131} Formation by Thyroid Mitochondria—Many of the agents which inhibit organic iodine formation in thyroid tissue slices also affect the particulate fractions of

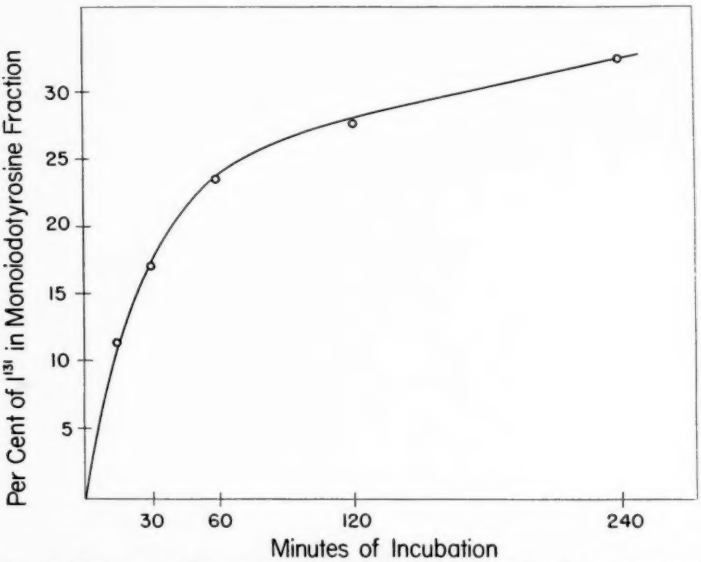


FIG. 4. Time-curve of formation of monoiodotyrosine-I^{131} by sheep thyroid mitochondria incubated with iodide-I^{131} in Krebs-Ringer bicarbonate solution.

thyroid (Fig. 6). Boiling the thyroid mitochondria completely abolished organic I^{131} formation (Fig. 6, *a*), as did also the presence of 10^{-3} M thiouracil (Fig. 6, *b*), 10^{-3} M thiocyanate (Fig. 6, *c*), or 10^{-3} M cyanide. The exclusion of oxygen from the medium and from the atmosphere also greatly reduced the formation of monoiodotyrosine-I^{131} (Fig. 6, *d*).

Addition of various cofactors (adenosinetriphosphate, cytochrome *c*, coenzyme I, succinate, coenzyme A, or thyrotropic hormone) to the mitochondrial preparations did not produce any consistent effects, except that coenzyme A was found to be strongly inhibitory. Perhaps the fact that coenzyme A contains a free sulfhydryl group, common to many goitrogens, caused this compound to interfere with organic iodine formation.

Iodination of Added Tyrosine by Whole Thyroid Homogenates and by Mitochondrial Suspensions—When tyrosine (0.003 M) was added to a sheep thyroid homogenate incubated with iodide-I^{131}, very little, if any, free monoiodotyrosine-I^{131} was formed. The further addition of $CuCl_2$ (0.0025 M) increased the quantity of free monoiodotyrosine-I^{131} formed, as previously reported by Fawcett and Kirkwood (4). In contrast with the

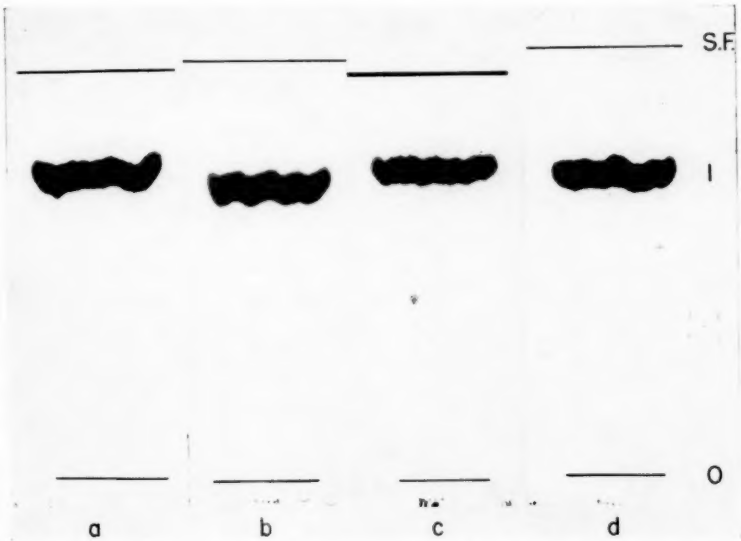


FIG. 5. Radioautographs of chromatograms of rat liver and kidney (cortex) mitochondrial and nuclear preparations incubated with iodide-I^{131} for 2 hours. *a*, liver mitochondria; *b*, liver nuclei; *c*, kidney mitochondria; *d*, kidney nuclei.

results reported by these workers, however, both mono- and diiodotyrosine-I^{131} were formed in our experiments (Fig. 7). When the concentrations of added copper and tyrosine were reduced to 0.001 M, the iodination of free tyrosine was greatly diminished.

The presence of copper and tyrosine had noticeable effects on the *endogenous* iodine metabolism of these thyroid preparations (Fig. 7). In both the homogenate and the mitochondria, but especially in the latter, the formation of the unidentified component with the high R_F value was greatly inhibited. The effect on the formation of bound monoiodotyrosine-I^{131} (as indicated by the radioactivity which remained at the origin of the chromatogram) differed in the two tissue preparations. It was enhanced in the whole homogenate, but reduced in the mitochondria.

XUM

Iodine and Nitrogen Contents of Mitochondrial and Nuclear Fractions—The analytical results are summarized in Table II. Each homogenate preparation represents the pooled thyroids of a dozen or more sheep slaughtered at the abattoir on a given day. There is a wide variation in the iodine contents of the different homogenates, probably related to the

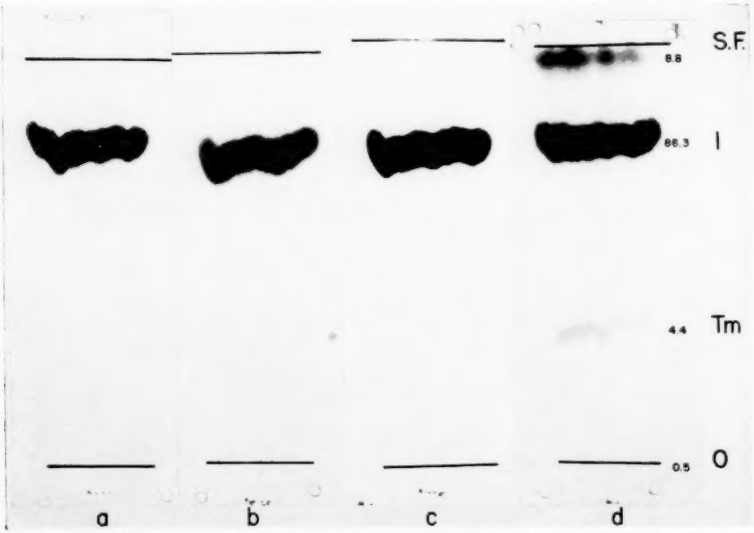


FIG. 6. Radioautographs of chromatograms of sheep thyroid mitochondria incubated with iodide-I^{131} under various conditions. *a*, boiled mitochondria used for incubation; *b*, 10^{-3} M thiouracil present during the incubation; *c*, 10^{-3} M thiocyanate present during the incubation; *d*, anaerobic conditions present during the incubation (compare with Fig. 2, *b*). All samples hydrolyzed with pancreatin for 8 hours before chromatography. See Fig. 1 for the explanation of numbers and symbols.

geographical origin and diet of the sheep. The nitrogen contents of the homogenate, however, are fairly uniform.

The soluble fraction of the gland contained by far the largest part of the total iodine (90 to 95 per cent). It also contained the highest concentration of iodine, expressed per mg. of nitrogen. The thyroid nuclei, on the other hand, contained the smallest part of the total iodine, in some instances much less than 1 per cent. The concentration of iodine in the nuclei was also much less than that in the other fractions in three of the four preparations tested. Although these results represent only total iodine analyses, they can hardly be reconciled with those of Rerabek (12), who reported that both the diiodotyrosine and thyroxine contents of thyroid nuclei are considerably higher than those of thyroid mitochondria

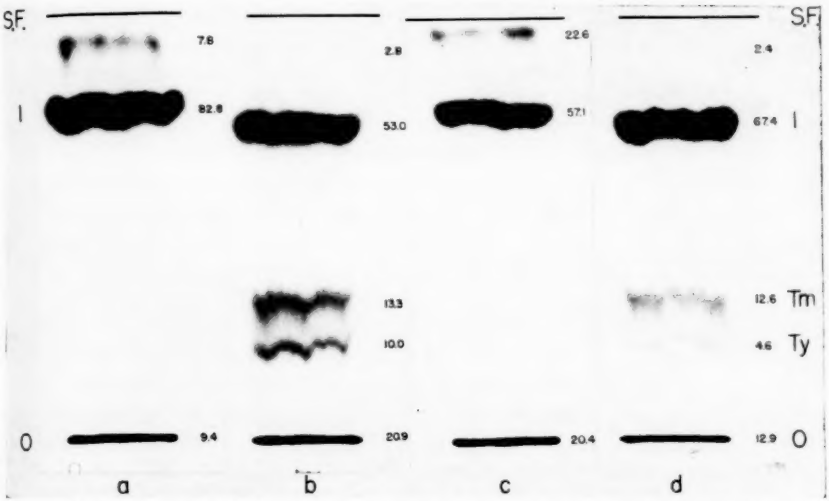


FIG. 7. Effect of added tyrosine and copper on formation of organic I^{131} by sheep thyroid homogenate and mitochondria incubated with iodide-I^{131} for 2 hours. Radioautographs of chromatograms of (*a*) whole thyroid homogenate, unhydrolyzed; (*b*) same homogenate as (*a*) incubated with I^{131} in presence of 0.003 M tyrosine and 0.0025 M $CuCl_2$, unhydrolyzed; (*c*) thyroid mitochondria, unhydrolyzed; (*d*) same mitochondrial preparation as (*c*) incubated with I^{131} in the presence of 0.003 M tyrosine and 0.0025 M $CuCl_2$, unhydrolyzed.

TABLE II

Nitrogen and Iodine Contents of Thyroid Homogenates and of Cell Fractions Derived from Them

Each cc. of homogenate represents approximately 150 mg. of sheep thyroid tissue.

Homogenate preparation No.	Whole homogenate		Mitochondrium-microsome fraction				Nuclear fraction				Soluble fraction			
	Mg. N per cc.*	γ I per cc.*	Mg. N per cc.*	γ I per cc.*	Per cent of total N	Per cent of total I	Mg. N per cc.*	γ I per cc.*	Per cent of total N	Per cent of total I	Mg. N per cc.*	γ I per cc.*	Per cent of total N	Per cent of total I
1	3.40	140	1.30	13.6	13	3.3	0.77	1.5	7.5	0.35	2.74	140	78	95
2	3.02	66	1.14	9.8	14	5.5	0.82	1.25	8.9	0.60				
3	3.62	180	1.27	20.4	12.5	4.0	0.99	16.8	8.6	2.9				
4	3.03	22.0	1.54	2.8	18.7	4.7	1.18	0.9	13.8	1.45				

* Refers to the particular suspension or solution used for incubation with iodide-I^{131}. The manner in which these were prepared is described under "Experimental."

XUM

in swine. Whether the discrepancy is due to species difference, to the different techniques used in fractionating the cell, or perhaps to the non-specific method used by Rerabek for determining iodine compounds remains to be investigated.

DISCUSSION

The results obtained in this investigation do not support the view that copper plays an essential rôle in the formation of organic iodine by the thyroid gland. Although we were able to confirm the findings of Weiss (3) and of Fawcett and Kirkwood (4), that added tyrosine can be iodinated by thyroid homogenates in the presence of cupric ion, we question the physiological significance of this observation. In the experiments reported here, whole thyroid homogenate and its particulate fractions, especially thyroid mitochondria, converted appreciable quantities of iodide-I^{131} to monoiodotyrosine-I^{131} in the absence of any added substrate. The monoiodotyrosine-I^{131} formed under these conditions was stably bound to protein, an observation in accord with other evidence that iodination of tyrosine takes place within a protein molecule (13). No organic I^{131} was formed in the cell-free systems if 10^{-3} M thiouracil, thiocyanate, or cyanide was present, or if the tissue preparation were first boiled for a few minutes. Furthermore, the soluble fraction of the thyroid homogenate, which contained by far the largest part of the gland's iodine, formed no organic I^{131} whatsoever when incubated with iodide-I^{131}. It appears, therefore, that some enzyme system is involved in the reaction studied here, either in the oxidation of iodide, or in the iodination of tyrosine, or in both. The possibility of an exchange reaction between an *oxidized* form of iodide-I^{131} and monoiodotyrosine is not completely excluded.

The physiological significance of the enzyme "tyrosine iodinase," reported by Fawcett and Kirkwood to be present in the thyroid and salivary tissue (5), remains to be determined. According to these workers, this enzyme catalyzes a monoiodination of *free* tyrosine by iodine, the latter being formed (in their experiments) by the oxidation of iodide by cupric ion. The thyroid homogenates which they used, however, formed only negligible amounts of *protein-bound* I^{131} in the absence of copper and tyrosine, a result not in agreement with our findings. We have no explanation for this discrepancy. The question whether tyrosine is iodinated in the thyroid gland as the free amino acid or as part of the thyroid protein requires further exploration. Our evidence favors the latter mechanism.

The nature of the unidentified component which moves almost with the solvent front and which was best demonstrated in experiments with thyroid mitochondria is of special interest. One possibility is that it represents an oxidized form of iodide which carries out the actual iodination of

the tyrosine. The following information is available regarding this component: (1) It appears primarily in homogenates and in particulate fractions rather than in thyroid tissue with intact cellular structure. This is consistent with the view that it is an iodinating agent whose turnover rate is extremely rapid in the intact thyroid cell. (2) It is not firmly bound to protein (*i.e.*, in peptide linkage), since it is present in chromatograms of unhydrolyzed material. (3) It almost always shows "tailing" after filter paper chromatography and appears, therefore, to be chemically unstable. (4) It is not a simple form of oxidized iodide (I_2 or HIO), since it is not reduced by thiouracil or thiosulfate. However, it is possible that I_2 may be bound to some organic molecule in thyroid tissue in such a way that it is not readily reduced. The actual iodinating agent in the thyroid gland may not be simply inorganic I_2, but rather an organic I_2 compound of some unknown type. This could explain the fact that, although an oxidized form of inorganic iodine is always assumed to be present in the thyroid gland, it has not been possible to demonstrate this directly (14).

All the chromatograms obtained during this investigation were prepared from mixtures containing added thiouracil (approximately 0.001 M). We have found this to be an effective means of eliminating artifacts which frequently appear on radioautographs of chromatograms of thyroid material. Most likely these artifacts occur when minute amounts of iodine-I^{131} are formed at some stage of the analytical procedure, either during enzymatic hydrolysis or during the chromatography. Thiouracil, because of its reducing properties, prevents the oxidation of inorganic iodide to the much more reactive iodine, while, at the same time, it has a negligible effect on organic I^{131} compounds which already have been formed. Even the radioactive iodide as received from Oak Ridge usually shows weak extraneous bands upon chromatography, but these are readily eliminated by the addition of thiouracil (or other reducing agents, such as thiosulfate or sulfite). The disadvantage involved in adding thiouracil to thyroid mixtures before chromatography is that simple oxidized forms of iodide which may be real intermediates in iodine metabolism are eliminated. However, in many experiments carried out without the addition of thiouracil, we were unable to demonstrate the presence of such intermediates.

SUMMARY

1. Whole thyroid homogenate and its various cellular fractions were investigated for ability to form organic I^{131} from added iodide-I^{131}. In the absence of any added substrate or oxidizing agent, the whole homogenate and the mitochondrial fraction were able to form appreciable quantities of monoiodotyrosine-I^{131}, as revealed by paper chromatography. The mitochondrial fraction was the most active per mg. of nitrogen. The sol-

uble fraction was completely inactive. The nuclear fraction showed some activity, but this may possibly have been due to contamination by whole cells or mitochondria.

2. Chemical analysis of the various cellular fractions of the thyroid indicated that the soluble fraction contained the largest portion of the total iodine (I^{127}). The nuclear fraction contained very little iodine, usually much less than the mitochondrial fraction.

3. The monoiodotyrosine-I^{131} which was formed by these tissue preparations was firmly bound to thyroid protein. It was released only after hydrolysis of the protein. Very little diiodotyrosine and practically no thyroxine were formed.

4. An unidentified I^{131} component was observed on the filter paper chromatograms of the homogenate and particulate fractions, especially in the mitochondrial preparations. The possible significance of this component is discussed.

5. Added copper and tyrosine are not necessary for the formation of monoiodotyrosine-I^{131} by thyroid homogenates and cell fractions.

6. The addition of thiouracil (approximately 10^{-3} M) to thyroid preparations before hydrolysis and paper chromatography is an effective means of eliminating artifacts which frequently appear on the chromatograms of such material.

BIBLIOGRAPHY

1. Morton, M. E., and Chaikoff, I. L., *J. Biol. Chem.*, **147,** 1 (1943).
2. Taurog, A., Chaikoff, I. L., and Franklin, A. L., *J. Biol. Chem.*, **161,** 537 (1945).
3. Weiss, B., *J. Biol. Chem.*, **201,** 31 (1953).
4. Fawcett, D. M., and Kirkwood, S., *J. Biol. Chem.*, **205,** 795 (1953).
5. Fawcett, D. M., and Kirkwood, S., *J. Biol. Chem.*, **209,** 249 (1954).
6. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and tissue metabolism, Minneapolis, 119 (1949).
7. Taurog, A., Tong, W., and Chaikoff, I. L., *J. Biol. Chem.*, **184,** 83 (1950).
8. Tong, W., Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **207,** 59 (1954).
9. Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **163,** 313 (1946).
10. Stanley, P. G., *Nature*, **171,** 933 (1953).
11. Roche, J., Michel, R., and Volpert, E., *Compt. rend. Soc. biol.*, **148,** 21 (1954).
12. Rerabek, J., *Biochim. et biophys. acta*, **8,** 389 (1952).
13. Tong, W., Taurog, A., and Chaikoff, I. L., *J. Biol. Chem.*, **191,** 665 (1951).
14. Fawcett, D. M., and Kirkwood, S., *J. Biol. Chem.*, **204,** 787 (1953).

STUDIES ON CARBOHYDRATE METABOLISM IN RAT LIVER SLICES

IV. BIOCHEMICAL SEQUENCE OF EVENTS AFTER INSULIN ADMINISTRATION*

By ALBERT E. RENOLD,† A. BAIRD HASTINGS, FRANCES B. NESBETT, AND JAMES ASHMORE‡

(From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts)

(Received for publication, September 14, 1954)

Abnormalities of hepatic carbohydrate metabolism in diabetic animals have been repeatedly described. We have previously reported that the presence of diabetes, following either alloxan administration or partial pancreatectomy, results, in rat liver slices, in a diminished glucose uptake, an almost completely inhibited glycogen deposition from glucose, and in a markedly increased formation of glucose from pyruvate, from glycogen, from fructose, and from unknown precursors (1, 2).

The primary site of the metabolic defect in diabetes is not known, though much evidence points to the initial steps of glucose utilization. Cori, working mainly with muscle, showed that decreased glucose phosphorylation was a major metabolic lesion resulting from insulin deficiency (3). Levine *et al.* suggested that insulin increases the permeability of the muscle cell surface to glucose (4), and Park added further support to this concept (5). Chernick and Chaikoff postulated a "block" between glucose and glucose-6-phosphate in liver slices from alloxan-diabetic rats (6) and recent studies from this laboratory have confirmed and extended their findings (2). The comparative utilization and metabolic fate of glucose and fructose, present together in the medium and alternately labeled with C^{14}, enabled us to obtain a quantitative estimation of the system transforming extracellular free glucose into intracellular phosphorylated glucose. Liver slices from diabetic rats phosphorylated glucose at one-tenth to one-fourth the normal rate.

When insulin is administered *in vivo*, metabolic effects on the isolated liver can be demonstrated. Thus the administration of insulin to diabetic rats for 1 to 4 days before sacrifice has been shown to restore the measured

* This work was supported in part by the United States Atomic Energy Commission, the United States Public Health Service, Swift and Company, and the Eugene Higgins Trust through Harvard University.

† Postdoctoral Fellow of the United States Public Health Service, 1951–53.

‡ Schering Fellow of the Endocrine Society, 1954.

diabetic metabolic abnormalities to normal (6–8). Knowledge of the biochemical sequence of events during this correction of diabetic metabolism by insulin might add information on the primary site or sites of insulin action. We have attempted to study this biochemical sequence of events within the limits of the measurements available to us.

Insulin was administered to diabetic rats for varying lengths of time before sacrifice; slices of their livers were incubated with labeled glucose, fructose, pyruvate, and acetate and analyzed. In addition, the diaphragms of these animals were incubated with labeled glucose and analyzed. It should be noted that, as on previous occasions, all incubations were carried out in a system designed to preserve a normal intracellular cationic environment (9).

Materials and Methods

Animals—Male albino rats of the Wistar strain, raised in our own colony, weighing between 250 and 350 gm. were used. They were fed *ad libitum* unless otherwise noted. The method employed in producing alloxan diabetes, as well as the criteria used to establish the presence of diabetes, has been previously described (1).

Medium and Substrates—Unless otherwise noted, the media used contained, in millimoles per liter, $K^+ = 110$, $Mg^{++} = 20$, $Ca^{++} = 10$, $HCO_3^- = 40$, $Cl^- = 130$; in the flasks containing acetate or pyruvate, $Cl^- = 90$ and $CH_3COCOO^- = 40$ or $CH_3COO^- = 40$. For the incubation of diaphragm, $K^+ = 110$ was replaced by $Na^+ = 110$ mmoles per liter. The solutions were equilibrated with 5 per cent CO_2-95 per cent O_2, giving a pH in the presence of liver slices varying between 7.4 and 7.5. C^{14}-labeled glucose[1] and fructose[1] were uniformly labeled in all carbons. Pyruvate[1] was labeled in the α position and acetate[1] was carboxyl-labeled. Each flask contained one substrate only, glucose and fructose in the concentration of 20 mmoles per liter and pyruvate and acetate in the concentration of 40 mmoles per liter.

Insulin—Glucagon-free insulin, specially prepared by the Nordisk Insulin Laboratorium and containing 26 units per mg., was used whenever insulin was added *in vitro* or given intravenously and subcutaneously within 3 hours of sacrifice. Squibb commercial protamine zinc insulin was used whenever a long acting preparation was needed.

Insulin Administration—In order to insure an immediate high and relatively constant insulin level in the diabetic animals from the time of injec-

[1] Uniformly labeled glucose and fructose were obtained from the Nuclear Instrument and Chemical Corporation, Chicago, Illinois. α-Carbon-labeled pyruvate and carboxyl-labeled acetate were prepared by Dr. Manfred Karnovsky of this laboratory.

tion to the time of sacrifice, even though these periods varied from 10 minutes to 48 hours, the following schedules of injection were adopted. Insulin administration was always begun by the intravenous injection of 10 units of crystalline, glucagon-free insulin. When the animals were sacrificed 10 minutes or 1 hour later, no other insulin was given. When they were to be sacrificed 6 hours after the beginning of insulin administration, an additional 5 units of glucagon-free crystalline insulin were administered subcutaneously 2 hours before sacrifice. When they were to be sacrificed after 24 or 48 hours of insulin action, 5 units of protamine zinc insulin were injected subcutaneously, together with the initial intravenous injection of crystalline insulin, and the injection of protamine zinc insulin was repeated every 12 hours; in addition 5 units of glucagon-free crystalline insulin were injected subcutaneously 2 hours before death.

Methods—Chemical methods and isotopic analyses used were similar to those previously described (1, 2, 9, 10). Fatty acids were isolated and prepared for isotopic analysis by the following modification of established procedures: The liver slices were subjected to alkaline hydrolysis and saponified with potassium hydroxide in 60 per cent ethanol, then extracted with petrol ether. The water-soluble residue was acidified to Congo red with hydrochloric acid, then reextracted with petrol ether. The petrol ether extract was washed with water until neutral, then evaporated to dryness in a boiling water bath. The residue was dissolved in alcohol, evaporated, redissolved in acetone-petroleum ether, and transferred to tared flasks, then evaporated and dried *in vacuo* over calcium chloride, potassium hydroxide, and paraffin. The final residue was weighed and a portion transferred to planchets, weighed, and counted. The average molecular weight of the fatty acids thus obtained was determined by titration and was found to correspond to an average of 12 carbon atoms per molecule.

Calculations—All calculations were identical with, or similar to, those previously described (1, 2, 9). From these we obtained glycogen synthesis from glucose, fructose, pyruvate, and acetate; CO_2 formation from glucose, fructose, pyruvate, and acetate; fructose disappearance from the medium and pyruvate disappearance from the medium. Also obtained were glucose uptake (1), glucose output (1), and total glucose phosphorylation (2); glucose formation from fructose, pyruvate, and acetate; incorporation of carbon from glucose, fructose, pyruvate, and acetate into fatty acids.

Results

Many attempts to demonstrate an effect of insulin on hepatic carbohydrate metabolism *in vitro* have been made in this laboratory. Although

it was thought in one series of experiments (11) that insulin, *in vitro*, had a demonstrable effect on glucose uptake and glucose output, an extension of the series has failed to confirm this preliminary conclusion. Indeed, as of the present time, we have been unable to establish a consistent, reproducible effect of insulin *in vitro*, and therefore no detailed account of these experiments will be given. In brief, insulin has been added in concentrations of up to 1 unit per ml. and, to insure good initial penetration of the hormone, whole liver and single liver lobes were on occasion perfused with insulin solutions before slicing. Measurements made included uptake of glucose, fructose, and pyruvate, as well as glycogen deposition and CO_2 production from these substrates, total glucose output, glucose production from fructose and pyruvate, and total glucose phosphorylation (2). In-

TABLE I

Measurements of Total Glucose Phosphorylation by Rat Liver Slices at Varying Time Intervals after Insulin Administration

No. of experiments	Animals	Total glucose phosphorylation (per cent of normal)
7	Normal	100 ± 6.2
7	Diabetic	6 ± 1.8
7	" given insulin intravenously from 10 to 70 min. before sacrifice	7 ± 2.1
5	Diabetic, given insulin intravenously and subcutaneously for 24 to 72 hrs. before death	87 ± 16.3

sulin was added to liver slices from normal, from both mildly and severely diabetic, and from adrenalectomized, hypophysectomized, and diabetic hypophysectomized rats. In no instance was a consistently positive effect obtained.

Effects of Insulin Administered in Vivo—Experiments were first performed to confirm the return towards normal of diabetic, hepatic, carbohydrate metabolism after 48 hours of insulin administration and to establish whether any early insulin effects, within minutes to 1 hour, could be demonstrated. Effects of the latter type can easily be shown in muscle (12), whereas Chernick and Chaikoff had noted that subcutaneous insulin administration to three diabetic rats for 1 to 3 hours before sacrifice did not restore glucose oxidation to CO_2 or glucose incorporation into fatty acids to normal (7). In our experiments, no early insulin effects on hepatic metabolism could be found, and the data concerning total glucose phosphorylation (2), the most sensitive index of glucose utilization available in our system, are summarized in Table I. It is evident that hepatic

glucose phosphorylation was markedly decreased in diabetic animals, and furthermore that 24 to 72 hours of insulin administration restored glucose phosphorylation to near normal activity. The intravenous administration of 5 to 10 units of insulin 10 to 70 minutes before sacrifice, however, did not affect the rate of glucose phosphorylation, even though this dose was sufficient to produce a drop in blood glucose from a mean of 447 mg. per cent to a mean of 231 mg. per cent between the time of insulin administration and sacrifice. The further addition of insulin *in vitro* after slicing was without effect. It might further be noted that, similarly, no early insulin effects on liver slices from five normal animals could be demonstrated.

TABLE II

Glycogen Deposition from Various Substrates by Rat Liver Slices and by Rat Diaphragm at Varying Time Intervals after Insulin Administration

All values expressed as micromoles of glucose equivalents per gm. of wet liver per 90 minutes.

Experiment No.	Animals	Duration of insulin administration	Liver glycogen from				Muscle glycogen from glucose
			Glucose	Fructose	Pyruvate	Acetate	
		min.					
1	Normal	0	22.2	36.2	10.8	0.05	4.0
2	"	0	17.4	27.4	3.6	0.01	5.2
3	"	0	14.0	24.0	8.4	<0.01	6.7
4	Diabetic	0	0.1	5.2	2.0	0.08	2.8
5	"	0	0.1	1.4	0.8	0.01	3.5
6	"	0	0.1	3.8	1.2	<0.01	4.8
7	"	10	0.1	5.7	0.7	0.2	20.2
8	"	10	0.1	5.0	3.0	<0.01	14.6
		hrs.					
9	"	1	0.2	2.0	1.2	0.01	11.5
10	"	1	0.2	4.9	3.5	0.05	9.0
11	"	1	0.2	6.7	1.3	<0.01	21.4
12	"	6	0.5	3.3	0.6	<0.01	2.0
13	"	6	0.4	8.1	2.8	0.04	10.0
14	"	24	12.5	12.7	2.1	<0.01	3.0
15	"	48	23.4	15.0	0.6	<0.01	3.2
16	"	48	28.0	22.0	1.6	<0.01	3.2

A more complete survey of the sequence of insulin effects in time seemed indicated and sixteen experiments were carried out. The results obtained are shown in Tables II, III, and IV. Some comments are warranted.

As shown in Table II, significant amounts of glycogen were synthesized from glucose, fructose, and pyruvate, but not from acetate. The presence of diabetes resulted in a markedly decreased hepatic glycogen synthesis

from all substrates, glycogen synthesis from glucose, however, being affected to a much more striking degree. The administration of insulin was without effect on glycogen synthesis from glucose up to 1 hour after insulin administration, but showed minimal effects after 6 hours, and restored glycogen synthesis to normal between 24 and 48 hours after the first insulin injection. The ratio, glycogen from glucose to glycogen from fructose, was much less than unity in the diabetic rat and remained so up to 24 hours

TABLE III

Incorporation of Carbon from Various Substrates into CO_2 and Fatty Acids by Liver Slices at Varying Time Intervals after Insulin Administration

All values expressed as micromoles of carbon per gm. of wet liver per 90 minutes.

Experiment No.	Animals	Duration of insulin administration	CO_2 from				Fatty acid carbon from			
			Glucose	Fructose	Pyruvate	Acetate	Glucose	Fructose	Pyruvate	Acetate
		min.								
1	Normal	0	51	119	177	58				
2	"	0	58	91	115	56	12.7	21.4	28.8	20.1
3	"	0	38	94	212	70				
4	Diabetic	0	6	137	152	59				
5	"	0	9	54	126	63	<0.1	0.5	0.9	1.1
6	"	0	4	87	140	49	<0.1	0.5	4.4	0.5
7	"	10	8	80	137	49	<0.1	0.6	0.9	0.2
8	"	10	6	79	218	83	<0.1	0.5	6.0	1.0
		hrs.								
9	"	1	7	59	182	61	<0.1	1.0	1.2	0.3
10	"	1	14	63	158	58	<0.1	0.3	4.3	0.8
11	"	1	7	107	189	59	<0.1	0.6	2.5	0.3
12	"	6	23	100	193	76		1.5	5.3	1.8
13	"	6	11	95	232	73	<0.1	0.5	7.3	0.7
14	"	24		100	174	52	10.0	10.7	43.5	24.6
15	"	48	87	97	132	23	46.0	28.6	130.0	25.2
16	"	48	92	101	139	29	62.0	61.0	176.0	35.7

after insulin administration, when it attained unity; it exceeded unity after. The same is true for the ratio of glycogen from glucose to glycogen from pyruvate, although to a different degree. The glycogen synthesis by muscle will be discussed later.

The presence of diabetes also resulted in a markedly decreased CO_2 production from glucose, whereas CO_2 production from fructose, pyruvate, and acetate remained unaffected (Table III). The administration of insulin was without effect up to 1 hour, showed minimal effects after 6 hours, and marked effects 48 hours after the beginning of insulin injection. As further shown in Table III, there were striking changes in the incorpora-

tion of substrate carbon into fatty acids. Diabetes resulted in a marked decrease of fatty acid synthesis from all sources, although the synthesis from glucose was decreased to a particularly marked degree. No significant insulin effect could be detected before 24 hours after the beginning of insulin administration. After 48 hours fatty acid synthesis from all substrates was not only normal but considerably more active than normal,

TABLE IV

Fructose Utilization, Total Glucose Phosphorylation, and Glucose Formation from Various Substrates by Rat Liver Slices at Varying Time Intervals after Insulin Administration

All values expressed as micromoles of fructose or glucose per gm. of wet liver per 90 minutes.

Experiment No.	Animals	Duration of insulin administration	Fructose utilized	Glucose phosphorylation			Glucose from		
				Maximum	Minimum	Mean	Fructose	Pyruvate	Acetate
		min.							
1	Normal	0	144	89	56	72	55	15	<1
2	"	0	139	88	53	70	57	10	<1
3	"	0	138	80	37	59	40	18	<1
4	Diabetic	0	108	2	2	2	77	26	<1
5	"	0	100	7	5	6	66	27	<1
6	"	0	112	3	2	2	61	20	<1
7	"	10	113	2	2	2	99	22	<1
8	"	10	138	3	2	2	82	35	<1
		hrs.							
9	"	1	110	11	7	9	75	32	<1
10	"	1	119	5	3	4	65	35	<1
11	"	1	136	4	2	3	63	20	<1
12	"	6	145	22	16	19	102	34	<1
13	"	6	130	7	5	6	90	45	<1
14	"	24	145	145	119	132	106	22	<1
15	"	48	115	178	141	160	76	9	<1
16	"	48	122	149	111	130	69	4	<1

this being particularly true for fatty acid synthesis from pyruvate and from glucose.

The data concerning hexose balance are shown in Table IV. Fructose utilization proceeded at approximately the same rate throughout. Total glucose phosphorylation, on the other hand, was almost negligible in the diabetic liver and was but gradually restored after insulin administration. Glucose synthesis from pyruvate was considerably more active in diabetic than in normal liver. It is of particular interest to note that this excessive gluconeogenesis from pyruvate was still present 24 hours after the beginning

of insulin administration and only reached normal activity or better between 24 and 48 hours after the first insulin injection.

In summary then, the administration of insulin to the diabetic animals resulted in an almost complete return to normal of all abnormal measurements within 24 to 48 hours. Indeed, some measurements (total glucose phosphorylation, CO_2 production from glucose, glucose synthesis from pyruvate, and fatty acid synthesis from all substrates) were overcorrected, thus indicating some insulin overdosage. The most striking observation, however, concerned the shorter periods of insulin administration. No insulin effects of any kind on hepatic carbohydrate metabolism were apparent at 10 minutes and 1 hour after the intravenous injection of insulin (although at 10 minutes the blood glucose level had already fallen from a mean of 435 mg. per cent to a mean of 357 mg. per cent, and at 1 hour from a mean of 426 mg. per cent to a mean of 217 mg. per cent). 6 hours after the beginning of insulin administration, there were suggestive but quantitatively small effects on glycogen synthesis from glucose, on CO_2 formation from glucose, and on total glucose phosphorylation. Glucose formation from pyruvate was still in the diabetic range 24 hours after the beginning of insulin administration.

The sequence of events in hepatic tissue after insulin administration was in marked contrast to the situation in striated muscle. Stadie *et al.* (12, 13) had previously shown that marked insulin effects result from exposure of diaphragm to insulin for a few minutes and Bleehen and Fisher (14) have demonstrated insulin effects on the perfused rat heart within 15 minutes of the addition of insulin to the system. Similarly, when the diaphragms of those animals, in which the hepatic metabolic changes have been described, were removed and incubated, maximal insulin effects were demonstrated within 10 to 60 minutes of insulin administration. The measurements of glycogen synthesis from glucose are shown in Table II. It is evident that glycogen synthesis from glucose was only moderately decreased in the diabetic muscle, and that insulin administration produced a marked increase in glycogen synthesis over and above normal values. Insulin effects were particularly marked 10 minutes and 1 hour after insulin administration; they were not apparent after 24 to 48 hours.

DISCUSSION

The effects of insulin on isolated liver and muscle, with respect to time, have been illustrated by three curves (Fig. 1).

Curve A represents the effect of insulin on the synthesis of glycogen from glucose by diaphragm. It is apparent that normal levels of glycogen synthesis from glucose are reached within minutes after the first administration of insulin, and that maximal insulin effects are reached within 10 to 60 minutes.

Curve B demonstrates the effect of insulin on total glucose phosphorylation by liver slices. This effect of insulin becomes apparent only gradually, normal values being reached between 6 and 24 hours and maximal values at 24 and 48 hours after the beginning of insulin administration. Similarly plotted curves representing hepatic glycogen synthesis from glucose and CO_2 formation from glucose follow the same general pattern.

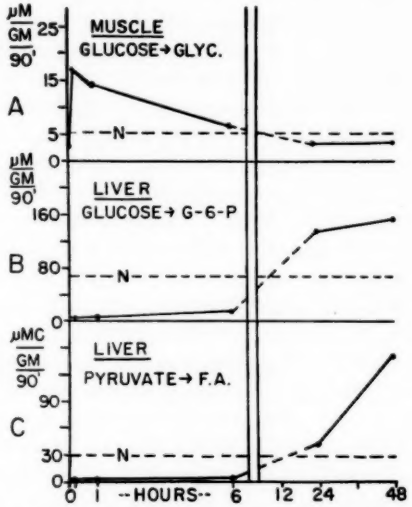


FIG. 1. Biochemical sequence of events after insulin administration to diabetic rats. Curve A, values expressed in micromoles of glucose equivalents per gm. of liver per 90 minutes. The dotted line represents the mean normal value. *Glyc.* = glycogen. Curve B, values expressed in micromoles of glucose per gm. of liver per 90 minutes. *G-6-P* = glucose-6-phosphate. Curve C, values expressed in micromoles of carbon per gm. of liver per 90 minutes. *F. A.* = fatty acids.

Curve C shows the effect of insulin on the synthesis of fatty acids from pyruvate. It would seem that the effect of insulin on fatty acid synthesis reaches its maximum somewhat later than the effect of insulin on total glucose phosphorylation. It is of interest to note that in adipose tissue the marked increase in lipogenesis from glucose which follows insulin administration (15) is similarly preceded by an increased glycogen deposition (16). The latter effect was shown to be, at least in part, a direct, local effect of the hormone on adipose tissue.

The most striking observation illustrated by these curves is the complete lack of early insulin effects on hepatic tissue, as opposed to the early and dramatic response of muscle. While this observation only applies to the metabolic functions measured, these included the utilization of four differ-

ent substrates, glucose, fructose, pyruvate, and acetate, their incorporation into glycogen, glucose, and fatty acids, and their oxidation to CO_2. Furthermore, it is difficult to question the adequacy of the insulin dosage used, in view of the marked effect obtained on both blood glucose levels and diaphragm. Three interpretations may be considered: (*a*) The metabolic functions measured may not have detected the primary site of insulin action on hepatic tissue. While this interpretation cannot be discarded, there is no positive evidence to support it. (*b*) Insulin was destroyed by hepatic tissue and therefore could not affect its metabolism. It is difficult to explain, however, why the destruction of insulin should be effective for a limited period of time only. (*c*) Insulin exerted immediate metabolic effects on the periphery only, mainly on muscle and perhaps adipose tissue (15, 16), while its effects on hepatic metabolism were slow and gradual, representing either a different type of hormonal action or a metabolic adaptation to the new demands created by the peripheral changes. This last interpretation seems to us the most likely one, and a brief discussion in the light of previous work may be warranted.

Convincing evidence of direct insulin effects on liver is scarce. Bouckaert and de Duve (17), however, measured in dogs the amount of glucose which had to be infused intravenously in order to maintain a constant blood glucose level in the presence of "maximal" insulin stimulation. After hepatectomy this glucose requirement decreased by about two-thirds. This was interpreted as indicating that about two-thirds of the total insulin effect on glucose disappearance was due to increased hepatic glucose uptake. This quite generally accepted interpretation was recently questioned by Lang, Goldstein, and Levine (18), who demonstrated that, under the conditions of Bouckaert and de Duve's experiments, hepatectomy is followed by decreased *peripheral* glucose uptake, and that it is not necessary to assume a direct hepatic insulin effect.

Bloch and Kramer (19), and later Brady and Gurin (20), as well as Haugaard and Stadie (21), have reported that insulin *in vitro* increases the synthesis of fatty acids from labeled acetate by liver slices from normal rats. However, no such insulin effect on liver slices from diabetic rats could be demonstrated *in vitro*. The present results are in accord with the latter observation.

At the present time it would seem reasonable, therefore, to retain the hypothesis that insulin exerts immediate metabolic effects at the periphery only. These metabolic effects reach their maximum so promptly that a physical action of insulin at the cell surface or activating effects on one or several superficially placed enzymes are suggested. On the other hand, the delayed and gradual effect of insulin on hepatic metabolism provides sufficient time for metabolic adaptation, including synthesis of new en-

zyme molecules, to occur. This could be the result of a direct, though slow, hormonal effect, quite different in type from the immediate peripheral action; it could also, however, represent adaptation to changes in substrate concentrations resulting from the changes in peripheral metabolism.

SUMMARY

1. Alterations of carbohydrate metabolism in liver slices and in diaphragm, after the administration of insulin to diabetic rats for varying periods of time, have been studied. The liver slices were incubated in a system designed to preserve a normal intracellular cationic environment.

2. The effects of insulin on diaphragm were apparent within a few minutes of its intravenous injection.

3. In contrast, no early insulin effects on hepatic carbohydrate metabolism could be demonstrated. Only minimal effects were noted 6 hours after the beginning of insulin administration, while normal values were reached after 24 to 48 hours. No consistent effects of insulin added *in vitro* could be shown.

4. The measurements made included the utilization of labeled glucose, fructose, pyruvate, and acetate, and the incorporation of each of these substrates into glycogen, glucose, CO_2, and fatty acids. The effects of insulin on lipogenesis and on gluconeogenesis appeared somewhat later than those on glucose phosphorylation.

5. These findings were thought to be consistent with the interpretation of immediate metabolic effects of insulin at the periphery only. The gradual effects on hepatic metabolism could be the result either of a direct, but slow, hormonal effect or of a metabolic adaptation to the new demands created by the peripheral changes.

BIBLIOGRAPHY

1. Renold, A. E., Teng, C.-T., Nesbett, F. B., and Hastings, A. B., *J. Biol. Chem.*, **204**, 533 (1953).
2. Renold, A. E., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **209**, 687 (1954).
3. Cori, C. F., *Harvey Lectures*, **41**, 253 (1945–46).
4. Levine, R., Goldstein, M., Klein, S., and Huddlestun, B., *J. Biol. Chem.*, **179**, 985 (1949).
5. Park, C. R., *J. Clin. Invest.*, **32**, 593 (1953).
6. Chernick, S. S., and Chaikoff, I. L., *J. Biol. Chem.*, **188**, 389 (1951).
7. Chernick, S. S., and Chaikoff, I. L., *J. Biol. Chem.*, **186**, 535 (1950).
8. Osborn, M. J., Chaikoff, I. L., and Felts, J. M., *J. Biol. Chem.*, **193**, 549 (1951).
9. Hastings, A. B., Teng, C.-T., Nesbett, F. B., and Sinex, F. M., *J. Biol. Chem.*, **194**, 69 (1952).
10. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **180**, 435, 447 (1949).
11. Hastings, A. B., Teng, C.-T., Nesbett, F. B., and Renold, A. E., *Federation Proc.*, **11**, 227 (1952).

XUM

12. Stadie, W. C., Haugaard, N., and Hills, A. G., *J. Biol. Chem.*, **184,** 617 (1950).
13. Stadie, W. C., *Physiol. Rev.*, **34,** 52 (1954).
14. Bleehen, N. M., and Fisher, R. B., *J. Physiol.*, **123,** 260 (1954).
15. Hausberger, F. X., Milstein, S. W., and Rutman, R. J., *J. Biol. Chem.*, **208,** 431 (1954).
16. Renold, A. E., Marble, A., and Fawcett, D. W., *Endocrinology*, **46,** 55 (1950).
17. Bouckaert, J. P., and de Duve, C., *Physiol. Rev.*, **27,** 39 (1947).
18. Lang, S., Goldstein, M. S., and Levine, R., *Am. J. Physiol.*, **177,** 447 (1954).
19. Bloch, K., and Kramer, W., *J. Biol. Chem.*, **173,** 811 (1948).
20. Brady, R. O., and Gurin, S., *J. Biol. Chem.*, **186,** 461 (1950).
21. Haugaard, E. S., and Stadie, W. C., *J. Biol. Chem.*, **200,** 753 (1953).

THE MECHANISM OF IRON RELEASE FROM FERRITIN AS RELATED TO ITS BIOLOGICAL PROPERTIES*

By ABRAHAM MAZUR, SILVIO BAEZ, AND EPHRAIM SHORR

(From the Department of Medicine, Cornell University Medical College, and The New York Hospital, New York, New York)

(Received for publication, July 22, 1954)

The function of ferritin as an iron storage protein has been established by a number of studies (1). Although an over-all hypothesis for iron transfer to and from ferritin depots has been suggested (2), little experimental evidence has been presented concerning the mechanisms involved. Investigations in this laboratory have revealed two other biological actions of ferritin: on intravenous injection (*a*) it inhibits the constrictor response to topical epinephrine of the precapillary sphincters and metarterioles in the mesenteric capillary bed of the rat (3) and (*b*) it inhibits urine flow in hydrated rabbits and dogs by stimulation of the neurohypophysis to secretion of its antidiuretic hormone (4). Our studies have also been concerned with the relationship of alterations in chemical structure of ferritin to its vascular and antidiuretic actions (5–7).

In the course of our studies we have obtained evidence that all three biological activities of ferritin involve the same chemical groups and the same mechanisms for their alteration. New evidence is here presented for the existence in ferritin of a dissociable form of iron, in the ferrous state, and for mechanisms in the liver which bring about its oxidation and reduction *in vitro*. Evidence for the operation of similar mechanisms in the intact animal is furnished by experiments in which liver hypoxia, induced by hemorrhage, leads to an increase in plasma iron, presumably from liver ferritin, in amounts sufficient to reduce profoundly the iron-binding capacity of the plasma. These studies provide the basis for suggestions as to some of the chemical mechanisms involved in the transport of iron in the body.

EXPERIMENTAL

Methods—Crystalline ferritin was prepared from horse spleen by methods previously published (3). It was recrystallized four times with $CdSO_4$,

* Aided by research grants from the Josiah Macy, Jr., Foundation, the National Institutes of Health, United States Public Health Service (grant H-79), the Armour Laboratories, the Research and Development Division, Office of the Surgeon General, Department of the Army (contract No. DA-49-007-MD-388), and the Postley Fund.

dissolved in 2 per cent ammonium sulfate, dialyzed, and reprecipitated from solution four times at 0.5 saturation with ammonium sulfate. The final precipitate was suspended in a minimum of water, dialyzed against distilled water to remove the ammonium sulfate, filtered through a Seitz filter, and stored in sterile bottles in the refrigerator. Liver slices were prepared as for Warburg respiration studies and incubated in a Krebs-bicarbonate medium with a gas phase consisting of 95 per cent oxygen or nitrogen and 5 per cent carbon dioxide. Sulfhydryl groups in ferritin were determined by a modification of the method of Rosner (6, 8) with iodoacetamide. Although the results of this determination may not give the true sulfhydryl content of ferritin, the values are reproducible. It is the only method which can be applied to this highly colored protein, and the results have been used for comparative purposes only. Plasma iron was determined by the method of Kitzes *et al.* (9) and plasma iron-binding capacity by the method of Rath and Finch (10). The iron-binding protein from human plasma was obtained as a 25 per cent solution of Fraction IV-7, approximately 70 per cent pure, from the blood fractionation laboratory at Harvard University and as a dry powder of a similar fraction from the Cutter Laboratories, Berkeley, California. Assay for vasodepressor activity of ferritin was carried out by the rat mesoappendix technique (11).

Ferrous Iron in Ferritin—Aliquots of a ferritin solution, previously adjusted to pH 7.4 with dilute NaOH, were added to 1 ml. of a 0.2 per cent solution of α,α'-dipyridyl at pH 7.4, together with water to make 5 ml., and allowed to stand at room temperature for 10 minutes. An equal volume of a saturated solution of ammonium sulfate, previously adjusted to pH 7.4 with ammonia, was added and the mixture inverted several times. A blank tube contained 4 ml. of water and all of the other reagents. The standard contained 4 ml. of a solution of ferrous ammonium sulfate, together with all of the reagents. After centrifugation, the clear supernatant solutions were read in a Klett-Summerson photocolorimeter equipped with a 520 mμ filter. The data (Table I) demonstrate the presence of small quantities of ferrous iron capable of combining with the dipyridyl reagent even at pH 7.4 and also the increased dissociation of this iron as the ferritin is diluted in the presence of a constant amount of the iron-binding reagent. These results are attributed to a competition between ferritin and the reagent for the ferrous iron.

The quantity of ferrous iron of ferritin bound by dipyridyl at various pH values was also measured. The method was essentially the same as that used in the previous experiment, except that the concentrations of both ferritin and dipyridyl were kept constant and the pH was varied. The quantity of ferrous iron bound by dipyridyl increased with increasing acidity; the values, expressed in terms of micromoles of ferrous iron per millimole of total iron, rose from 0.20 at pH 9.5 to 1.34 at pH 2.0. These

results are a reflection of the increased dissociation of ferritin ferrous iron at low pH and possibly the denaturation by acid of ferritin (5), with a resultant uncovering of ferrous iron available for reaction with dipyridyl.

Relationship of Ferritin-Ferrous Iron and Sulfhydryl Groups—Rothen (12), on the basis of sedimentation studies with ferritin, concluded that this protein is a heterogeneous mixture of molecules of varying iron content, but with the same protein moiety, apoferritin. He also demonstrated the presence of apoferritin, essentially free of iron, in a preparation of crystalline ferritin. We have obtained similar results by analyses of the layers

TABLE I

Dissociation of Ferritin Iron by Dilution

Aliquots of a ferritin solution (5 mg. of N per ml.) were mixed with 1 ml. of 0.2 per cent α,α'-dipyridyl, pH 7.4, and varying amounts of water to give a total volume of 5 ml. 5 ml. of saturated ammonium sulfate solution, adjusted to pH 7.4, were added and the mixture centrifuged. The supernatant solutions were compared, in a photocolorimeter having a 520 mμ filter, with a similarly treated standard ferrous ammonium sulfate solution.

Ferritin concentration	$\frac{\text{Ferrous iron}}{\text{Total iron}}$
mg. N per 5 ml.	*μmole per mmole*
25.0	0.25
15.0	0.27
10.0	0.30
5.0	0.33
2.5	0.42
1.25	0.46
0.50	0.68

which result from high speed centrifugation of ferritin solutions, and also of fractions serially precipitated from solution by increasing amounts of ammonium sulfate (6). In the present study a similar fractionation with ammonium sulfate was performed, and each precipitate was thoroughly dialyzed before analysis for total iron, ferrous iron, and sulfhydryl groups. Before analyses were carried out, all fractions were adjusted to the same pH and to the same concentration of protein nitrogen to eliminate the factor of variable dissociation of ferrous iron which would occur at different pH values and protein concentrations. Table II lists the results obtained. The total iron of these fractions decreased as the concentration of ammonium sulfate required to precipitate them from the solution increased; that fraction precipitated at the highest concentration contained the least total iron. From Rothen's data one may assume that this fraction was relatively rich in apoferritin.

It is clear from our data that, although the total iron in the protein de-

creases progressively with increasing concentration of ammonium sulfate, the ferrous iron content remains essentially unchanged. The sulfhydryl groups in the protein of the various fractions also remain constant over a wide range of ammonium sulfate concentration, increasing only in that fraction rich in apoferritin. This would suggest an association between sulfhydryl groups and ferrous iron with an excess of sulfhydryl groups in the last fraction, as if the latter were unsaturated with respect to ferrous iron-binding capacity.

Effect on Ferritin-Ferrous Iron of Alteration of Sulfhydryl Groups—The presence in ferritin of sulfhydryl groups may serve to prevent the autoxidation of ferrous iron. It should be possible to demonstrate this either by

TABLE II

Relationship of Total Iron, Ferrous Iron, and Sulfhydryl Groups in Ferritin Fractions

A solution of purified ferritin was treated serially with increasing amounts of ammonium sulfate. The precipitates obtained were separated, dissolved in water, and dialyzed thoroughly. Before analysis they were adjusted by dilution to contain the same concentration of total nitrogen.

The results are in micromoles per millimole.

Concentration of $(NH_4)_2SO_4$	$\frac{\text{Total iron}}{\text{Total N}}$	$\frac{\text{Ferrous Iron}}{\text{Total N}}$	$\frac{\text{SH}}{\text{Total N}}$	$\frac{\text{Ferrous iron}}{\text{Total iron}}$
per cent of saturation				
Original	(454)	(0.8)	(4.1)	(1.7)
0–27	549	0.7	3.2	1.2
27–31	454	0.6	3.3	1.4
31–34	361	0.6	3.4	1.6
34–50	251	0.8	5.1	3.2

blocking the sulfhydryl groups with an alkylating agent or by oxidizing them to the disulfide state. Aliquots of a ferritin solution containing 15.0 μmoles of sulfhydryl per 100 mg. of ferritin N were treated with a 2-fold excess of *p*-chloromercuribenzoate, iodoacetamide, or *o*-iodosobenzoate and then dialyzed free of excess reagent. The first two are agents which combine specifically with sulfhydryl groups; the last is an oxidizing agent for sulfhydryl groups. The amounts used were sufficient to decrease the sulfhydryl content of the ferritin to zero, as measured by reaction with iodoacetamide. The untreated ferritin solution contained 1.4 μmoles of ferrous iron per 100 mg. of ferritin N, whereas each of the treated aliquots contained 0.5 μmole of ferrous iron per 100 mg. of ferritin N, a decrease to one-third of the original value. This result is particularly significant in the case of *p*-chloromercuribenzoate, because it is not an oxidizing agent and is highly specific for sulfhydryl groups.

Effect of Reducing Agents on Ferritin Iron—Table III demonstrates that treatment of ferritin with naturally occurring reducing agents such as reduced glutathione, ascorbic acid, or cysteine results in the liberation of relatively large quantities of ferrous iron. In each case equivalent concentrations of reducing agent were incubated with ferritin at pH 7.4 for 30 minutes. Cysteine was the most effective agent, ascorbic acid the next best. Aliquots of the mixtures were precipitated with $(NH_4)_2SO_4$ and then dialyzed against distilled water overnight with several changes of water. Analyses of the dialyzed protein revealed that the extra ferrous iron liberated by the reducing agents had been removed by dialysis, demonstrating the lability of the ferritin-ferrous bond and the effectiveness of the reducing agents as iron-binding compounds.

TABLE III

Effect of Reducing Agents on Ferritin-Ferrous Iron

8 ml. of a ferritin solution were mixed with 2 ml. of the reducing agent so that the final concentration of the reducing agent was 0.067 M and the pH 7.4. After 30 minutes at 37.5° an aliquot was analyzed for ferrous iron at pH 4.6. The ferritin in the remainder of the solution was precipitated at 0.5 saturation with ammonium sulfate, and the precipitate dissolved in water and dialyzed against water before analysis for ferrous iron.

Treatment	$\frac{\text{Ferrous iron}}{\text{Total iron}}$
	μmoles per mmole
Original ferritin	0.86
Before dialysis, (*a*) ferritin + GSH	9.3
" " (*b*) " + ascorbate	27.6
" " (*c*) " + cysteine	105.0
After dialysis, (*a*)	0.61
" " (*b*)	0.87
" " (*c*)	0.69

In an earlier study (6) we reported that iodoacetamide-treated ferritin, which had lost its vasodepressor activity, could be reactivated by treatment with an excess of a reducing agent such as cysteine or reduced glutathione. This finding was puzzling, since the reaction of iodoacetamide with sulfhydryl groups is not considered to be reversible.

Experiments were carried out to determine whether the iron of iodoacetamide-treated ferritin was altered by incubation with cysteine, ascorbic acid, or reduced glutathione. It is apparent (Table IV) that none of the three reducing agents was capable of restoring the original sulfhydryl content of the ferritin, whereas in all cases the ferrous iron content rose several fold above the original value. It may be pointed out that these

ferrous iron values are minimal, since much was undoubtedly lost during the dialysis required to remove the sulfhydryl reagents, cysteine and glutathione.

Although the analytical results in Table IV indicate some increase in sulfhydryl content as a result of incubation with glutathione (from 6.5 to 8.7 μmoles), it was suspected that this increase was due not to ferritin sulfhydryl groups but rather to protein-bound glutathione. Krimsky and Racker have shown (13) that glutathione is a firmly bound component of the enzyme glyceraldehyde-3-phosphate dehydrogenase. They and others (14, 15) have also shown that, even after preliminary treatment of the

TABLE IV

Effect of Reducing Agents on Ferrous Iron Content of Iodoacetamide-Treated Ferritin

A ferritin solution was allowed to stand for 10 minutes at room temperature at pH 7.4 with an amount of iodoacetamide equivalent to the sulfhydryl content of the protein. The ferritin was then precipitated with an equal volume of saturated ammonium sulfate and the precipitate dissolved and dialyzed against water to remove excess iodoacetamide and ammonium sulfate. Aliquots were incubated with equivalent concentrations of the reducing agents at pH 7.4 and then exhaustively dialyzed against a dilute phosphate buffer, pH 7.4. Analyses were carried out on the dialyzed solutions.

Ferritin treatment	$\frac{SH}{Total\ N}$	$\frac{Ferrous\ iron}{Total\ N}$
	μmoles per 100 mg. N	*μmoles per 100 mg. N*
(*a*) Original	16.5	2.0
(*b*) Ferritin + iodoacetamide	6.5	1.2
(*c*) (*b*) + cysteine	5.2	11.1
(*d*) " + ascorbate	5.7	4.9
(*e*) " + glutathione	8.7	8.4

enzyme with iodoacetic acid, it can react with glutathione and regain part of its activity. In the light of these findings 10 ml. of a solution of iodoacetamide-treated ferritin (7.44 mg. of ferritin N per ml.), which had been exhaustively dialyzed against water and which had no measurable sulfhydryl content, were incubated for 30 minutes at 37.5° at pH 7.4 with 1 ml. of a solution of S^{35}-labeled glutathione[1] (9.44 mg.). The mixture was dialyzed during a period of 4 days against frequent changes of water. The dialysis was interrupted when the outside solution contained low but constant amounts of radioactivity. The protein solution contained 3.0 μmoles of sulfhydryl groups per 100 mg. of ferritin N. Radioactivity analyses showed that the iodoacetamide-treated ferritin had bound the radioactive glutathione in amounts approximately equivalent to the ob-

[1] Schwarz Laboratories, Mt. Vernon, New York.

XUM

served increase in sulfhydryl content; *i.e.*, 4.3 μmoles of sulfhydryl per 100 mg. of ferritin N calculated from the bound S^{35}.

Effect of Liver Slices on Ferritin Iron—Previous studies have shown that ferritin-disulfide groups are reduced to the sulfhydryl state by anaerobic incubation with normal liver slices, whereas aerobic incubation results in a lowered sulfhydryl content (7). These experiments have now been repeated, with the inclusion of measurements of parallel changes in ferrous iron. As a control, liver slices were incubated in oxygen or nitrogen in the absence of ferritin and the medium was analyzed for ferrous iron. The anaerobic liver slices released more ferrous iron (9.0 γ per 5 ml.) into the

TABLE V

Effect of Liver Slices on Sulfhydryl Groups and Ferrous Iron of Ferritin

A ferritin solution containing the ingredients of a Krebs-bicarbonate solution, but without the phosphate, was incubated with one-fifth its weight of rat liver slices in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide for 2 hours at 37.5°. Aliquots were removed for analysis and for dialysis against a solution of iron-binding protein of human plasma of the same concentration as the ferritin solution and in the same medium. The remainder of the ferritin was incubated with fresh rat liver slices in a 95 per cent oxygen-5 per cent carbon dioxide atmosphere and then treated in the same way.

Ferritin treatment	SH	Fe^{++} (*c*)	Fe^{++} bound by plasma iron-binding protein	
			Total	Per cent of (*c*)
	μmoles per 100 mg. ferritin N	*μmoles per 100 mg. ferritin N*	*μmoles per 100 mg. ferritin N*	
Original ferritin	25.0	1.7	0.8	47
(*a*) Ferritin + liver slices in N_2	33.7	6.5	5.7	88
(*b*) (*a*) + liver slices in O_2	14.9	2.3	0.2	9

medium than did the aerobic slices (4.0 γ per 5 ml.). The origin of this iron is presumed to be ferritin present in the liver slices. Table V lists the results of analyses for sulfhydryl groups and ferrous iron of ferritin after anaerobic incubation with rat liver slices. Both sulfhydryl and ferrous iron values were increased. This reduced form of ferritin was then incubated aerobically with fresh rat liver slices, with a marked decrease in sulfhydryl groups as well as in ferrous iron.

The last column in Table V shows the extent of iron transfer when the various ferritins obtained in that experiment were dialyzed against a solution of human plasma iron-binding protein. The maximal transfer of ferrous iron across the membrane for combination with the iron-binding protein occurred with the ferritin which had been exposed to anaerobic

incubation with liver slices (88 per cent of the total ferrous iron present). The least transfer (9 per cent) took place with ferritin which had been aerobically incubated. The transfer of iron from the original ferritin was intermediate (47 per cent). These findings further suggest a direct relationship between the sulfhydryl content and the dissociability of the ferrous iron in ferritin.

Effect of Plasma Iron-Binding Protein on Vasodepressor Activity of Ferritin—Our earlier experiments had demonstrated that the vasodepressor activity of ferritin is dependent on the presence of free sulfhydryl groups (6). Experiments were now designed to explore the possible rôle of ferrous iron in this vasodepressor activity of ferritin. Ferritin solutions of known vasodepressor activity were incubated with iron-binding protein at pH 7.4 for 10 minutes at room temperature, and the mixture was diluted with saline for assay by the rat mesoappendix method. Untreated ferritin solutions gave strongly positive vasodepressor tests in amounts of 0.0005 and 0.0001 γ of ferritin N. The activity of both these quantities of ferritin was masked or destroyed by the presence of a final concentration of 0.01 to 0.1 per cent iron-binding protein. The iron-binding protein itself was without activity. These results suggest that the vascular action of ferritin is specifically dependent on its ferrous iron, which in turn owes its stability to the presence of sulfhydryl groups.

Plasma Iron of Dogs during Hemorrhage—The increase of labile ferrous iron of ferritin *in vitro* after anaerobic incubation with liver slices and the ability of this iron to dialyze across a membrane for combination with plasma iron-binding protein suggested that liver hypoxia *in vivo*, such as that which results from prolonged drastic hemorrhagic hypotension, should result in an increase of plasma iron and a simultaneous decrease of the plasma iron-binding capacity. To test this hypothesis a number of dogs were subjected to graded hemorrhage (16) in the following manner: The animals were bled at 15 or 30 minute intervals over a period of 75 to 165 minutes in amounts decreasing from 2 per cent of the body weight at the first bleeding to 0.2 per cent as the blood pressure level approached 40 mm. of Hg. They were then maintained at that blood pressure level for 100 to 160 minutes longer by judicious removal of blood or infusion of plasma. Plasma was used for infusions to avoid hemolysis. Heparinized blood samples were withdrawn at intervals, and the plasma was analyzed for iron as well as iron-binding capacity. At the conclusion of the experimental period all the blood which had been withdrawn was reinfused. This procedure resulted in death from irreversible shock within 24 hours in 64 per cent of the control group.

Table VI presents the essential details of a typical experiment with a dog which was bled to the stage of irreversible shock. During the early part

of the experiment, before the blood pressure was significantly reduced, the plasma iron and iron-binding capacity were relatively unaffected. With a decrease of blood pressure to drastic hypotensive levels, the plasma iron increased steadily, coincident with a decrease in plasma iron-binding capacity. At the end of the experiment the dog was infused with the blood previously withdrawn. The final blood sample taken during transfusion showed that the iron-binding protein of the plasma was virtually saturated with respect to iron. The dog died 12 hours later.

TABLE VI

Plasma Iron in Control Dog during Hemorrhage

Dog 387, weighing 12.7 kilos, was anesthetized with 3.5 mg. of sodium pentobarbital per kilo, bled by the standard technique, and infused with the blood withdrawn at the end of the experiment. It died 12 hours later.

Sample No.	Time	Blood pressure	Plasma protein	Plasma iron	Plasma iron-binding capacity	Saturation of iron-binding protein (calculated)
	min.	*mm. Hg*	*gm. per 100 ml.*	*γ per 100 ml.*	*γ iron per 100 ml.*	*per cent*
1	0	120	6.5	73	170	30
2	30	120	6.5	73	172	30
3	60	120	6.5	58	178	25
4	105	100	6.5	54	178	23
5	135	60	6.5	68	160	30
6	180	70	5.2	107	160	40
7	210	40	5.6	127	80	61
8	245	30		172	30	85
9	315	20				
10	345			275	<10	>97

A total of fourteen control dogs was bled by this standard procedure; nine of these died within 24 hours after transfusion. Their last blood samples showed marked increases in plasma iron (average 287 per cent of original plasma iron, range 173 to 534 per cent), marked decreases in plasma iron-binding capacity (average 15 per cent of original capacity, range 6 to 25 per cent), and marked increases in saturation of plasma iron-binding protein (average 90 per cent saturation, range 80 to 97 per cent, as contrasted with a range of 20 to 40 per cent saturation at the start of the experiment). Of the remaining five dogs, one, having plasma values close to these averages, died subsequently. The average values and the range for the four survivors indicated less marked changes (plasma iron 171 (142 to 216) per cent; iron-binding capacity 46 (38 to 58) per cent; saturation of iron-binding protein 70 (55 to 84) per cent).

Previous studies in our laboratory (17), confirming and extending the

results of others (18), have shown that pretreatment with the adrenergic blocking agent, Dibenzyline, affords a high degree of protection against the otherwise lethal outcome of the graded hemorrhagic procedure employed. Among the mechanisms involved in this protection is a better maintenance of blood flow through the abdominal organs, including the liver, as a result of the action of Dibenzyline in blunting peripheral vasoconstriction. Accordingly, six dogs were given Dibenzyline 17 hours prior to the experiment and then subjected to an equivalent degree and duration of hemorrhagic hypotension. In these Dibenzyline-treated dogs the plasma iron did not rise, nor was there any essential change in iron-binding capacity; consequently the saturation of the iron-binding protein was unaltered. The average plasma values and range were 100 (50 to 150) per cent of the original iron, 86 (54 to 133) per cent of the original iron-binding capacity, and 40 (18 to 67) per cent saturation of the iron-binding protein.

The results generally support the hypothesis that liver hypoxia leads to the formation of sulfhydryl-ferrous-ferritin and the release of its ferrous iron into the plasma, where it is bound by the iron-binding protein.

Effect of Anaerobic Bone Marrow on Ferritin Iron—The presence of ferritin in the bone marrow makes this protein a likely source of iron for heme synthesis. The combination of ferrous iron with protoporphyrin has been reported by Granick (19) to take place in the presence of chick nucleated red cell hemolysates under anaerobic conditions. Minced rabbit bone marrow was incubated with ferritin under anaerobic conditions in a manner similar to that used for liver slices. Measurement of ferritin-ferrous iron available for combination with dipyridyl showed increases comparable to those reported for liver; *e.g.*, from 0.84 to 2.52 expressed in terms of micromoles of ferrous iron per millimole of total iron. These results suggest that local conditions of hypoxia would favor the conversion of ferric to ferrous iron in bone marrow ferritin, with the release of ferrous iron for purposes of heme synthesis.

Presence of Ferritin in Human Placenta—An important aspect of iron transport is the movement of iron from the maternal to the fetal circulation. The finding (20),[2] made independently in our laboratory, that the human placenta contains ferritin may help to clarify the mechanism for such a transfer. We have isolated crystalline ferritin from fresh human placenta by methods previously described for liver and spleen (3). In three placentae estimations of the amount of ferritin were made by the quantitative immunochemical technique of Heidelberger as previously applied to dog tissues (22). By this method they were found to contain 6, 10, and 12 γ of ferritin N per gm. of wet weight of tissue.

[2] Latham, E. F., and Vosburgh, G. J., unpublished, referred to by Vosburgh and Flexner (21).

DISCUSSION

Our present data show that a small proportion of the total iron in reduced or sulfhydryl ferritin is present in the ferrous state at or near the surface of the protein. This iron is readily available for reaction with dipyridyl or the plasma iron-binding protein, indicating an ability to dissociate from ferritin in the presence of a suitable iron-binding agent. In contrast, the bulk of iron in ferritin appears to be present in the ferric state and unavailable for reaction with iron-binding agents. On the basis of results of viscosity, electrophoresis, and antigen-antibody reaction experiments with ferritin and apoferritin (5), we have postulated that most of the iron in ferritin must be internally situated, since it does not appear to affect the surface properties of the protein. The relatively small amounts of ferrous iron at the surface of ferritin would not affect these properties to any great degree at the pH of these measurements. Confirmation of the hypothesis of an internal structure for ferritin iron is furnished by the electron microscope studies of Farrant (23).

The presence of two forms of iron in ferritin suggests an equilibrium between the micellar iron inside the molecule and small quantities of ionic iron at the surface, an equilibrium which can be shifted by the action of reducing agents such as glutathione, which converts surface ferric to ferrous iron. Since the liver cell contains relatively high concentrations of glutathione, the latter may be the active constituent of liver slices which brings about the conversion of ferric-disulfide-ferritin to ferrous-sulfhydryl-ferritin under the stimulus of liver hypoxia, the iron being transferred to plasma for combination with the plasma iron-binding protein.

The simultaneous alteration of sulfhydryl groups and ferrous iron by liver slices or by sulfhydryl-oxidizing or alkylating agents and the direct relationship between these two chemical groups in the various molecules of crystalline ferritin make it highly likely that chelation of ferrous iron by the sulfhydryl groups of the protein is the mechanism by which its autoxidation is prevented, a process which does occur when inorganic ferrous iron is added to water or to most proteins at neutral or slightly alkaline pH.

Our data also reveal the nature of the groups in ferritin requisite for its biological activity. Ferritin is active as a vasodepressor and as an antidiuretic only when it contains free sulfhydryl groups as well as ferrous iron available for dissociation and reaction. Since the removal of surface ferrous iron from sulfhydryl ferritin abolishes its vascular action, ferrous iron may represent the group more specifically related to this biological activity. Accordingly, the sulfhydryl groups in ferritin would play the secondary rôle of stabilizing the ferrous iron. Conversely, the inactivation of ferritin by aerobic exposure to liver slices is associated with a conversion of sulfhydryl-ferrous-ferritin to the disulfide-ferric state. It is suggested that the re-

action ferric-disulfide-ferritin → ferrous-sulfhydryl-ferritin also represents a mechanism for the release of iron from its storage state. The results of the experiments on hemorrhagic shock illustrate the effects of liver hypoxia on this mechanism of iron release. In this instance, release from the hypoxic liver of iron to the plasma, which makes it ultimately available for the synthesis of new red cells, could serve as a compensatory response to the acute loss of blood. Since we have obtained similar results with a splenectomized dog, it may be assumed that the liver is the major source of extra plasma iron during hemorrhage.

The mechanism of transfer of ferritin iron to the iron-binding protein of the plasma is indicated by our dialysis experiments, which demonstrate that transfer of iron to the iron-binding protein takes place only from sulfhydryl-ferrous-ferritin. This observation is consistent with the fact that the plasma protein combines avidly with ferrous iron to form a complex at pH 7.4. The state of the iron in this protein complex is less certain. Although some workers assume that it is completely converted to the ferric state after combination (24–26), Laurell (27) reports data which make it probable that some, at least, is present in the ferrous form and that the reaction is reversible. The plasma iron-binding protein is the carrier of circulating iron to the bone marrow. There it could be released in the ferrous form, possibly with the aid of glutathione, for incorporation into ferritin stores in the marrow. The ability of anaerobic bone marrow to reduce ferritin iron to the ferrous state presents a mechanism for the release from bone marrow ferritin of iron for heme synthesis.

The finding of ferritin in human placenta strongly suggests that local intermittent hypoxia in the placenta results in the appearance of ferritin iron in the ferrous form for transfer across the placental barrier to the fetal blood stream. Evidence for the existence of hypoxic conditions in the placenta is provided by the findings of Walker and Turnbull (28).

The presence of ionic iron at or near the surface of ferritin which can be reduced by liver, bone marrow, and placenta offers a convenient mechanism for activation of the various biological properties of this protein: its vasodepressor effect, its antidiuretic effect, and its iron release properties. Although the evidence is not complete, it appears likely that the reverse reaction, the incorporation of iron into ferritin, requires oxidative conditions. A system for the conversion of ferrous to ferric iron in ferritin has been demonstrated to operate in aerobic liver slices. By this reaction vasodepressor, antidiuretic, and iron release properties of ferritin are lost and the protein assumes the rôle of iron storage.

The authors are indebted to Dr. B. Burack and Iris Forbes for help with the animal experiments and to Greta Sander for expert chemical assistance.

XUM

SUMMARY

Crystalline ferritin, isolated from anaerobic liver or spleen, contains small but measurable amounts of its iron in the ferrous state, as well as free sulfhydryl groups.

Oxidation or blocking of ferritin sulfhydryl groups by aerobic liver slices, *o*-iodosobenzoate, *p*-chloromercuribenzoate, or iodoacetamide leads to a decrease in ferritin-ferrous iron. Treatment of ferritin with anaerobic liver slices, glutathione, cysteine, or ascorbic acid leads to an increase in ferritin-ferrous iron. These data suggest an association between ferritin sulfhydryl groups and ferrous iron which serves to prevent the autoxidation of the latter.

The ferrous iron in sulfhydryl ferritin is dissociable at pH 7.4, as evidenced by the ease with which it is transferred across a membrane for combination with human plasma iron-binding protein. This reaction occurs *in vivo* during drastic hemorrhagic hypotension and results in the progressive transfer of ferrous iron from sulfhydryl-ferrous-ferritin in the hypoxic liver to the plasma, with a parallel reduction in the iron-binding capacity of the plasma.

The form of ferritin which is active both as a vasodepressor and antidiuretic contains both free sulfhydryl groups and ferrous iron. The removal of ferrous iron from sulfhydryl-ferrous-ferritin by reaction with plasma iron-binding protein abolishes its vasodepressor activity, suggesting that it is the ferrous iron rather than the sulfhydryl groups which is more specifically related to this physiological property.

A hypothesis is advanced for the rôle of ferritin in iron transport and heme synthesis based on the mechanisms described for the storage and release of ferritin iron by the oxidation and reduction, respectively, of this protein.

BIBLIOGRAPHY

1. Granick, S., and Hahn, P. F., *J. Biol. Chem.*, **155,** 661 (1944); *Science*, **103,** 107 (1946). Granick, S., *J. Biol. Chem.*, **164,** 737 (1946).
2. Granick, S., *Chem. Rev.*, **38,** 379 (1946).
3. Mazur, A., and Shorr, E., *J. Biol. Chem.*, **176,** 771 (1948).
4. Baez, S., Mazur, A., and Shorr, E., *Am. J. Physiol.*, **162,** 198 (1950); **169,** 123, 134 (1952).
5. Mazur, A., Litt, I., and Shorr, E., *J. Biol. Chem.*, **187,** 473 (1950).
6. Mazur, A., Litt, I., and Shorr, E., *J. Biol. Chem.*, **187,** 485 (1950).
7. Mazur, A., Litt, I., and Shorr, E., *J. Biol. Chem.*, **187,** 497 (1950).
8. Rosner, L., *J. Biol. Chem.*, **132,** 657 (1940).
9. Kitzes, G., Elvehjem, C. A., and Schuette, H. A., *J. Biol. Chem.*, **155,** 653 (1944).
10. Rath, C. E., and Finch, C. A., *J. Clin. Invest.*, **28,** 79 (1949).
11. Zweifach, B. W., in Potter, V. R., Methods in medical research, Chicago, **1,** 131 (1948).

12. Rothen, A., *J. Biol. Chem.*, **152,** 679 (1944).
13. Krimsky, I., and Racker, E., *J. Biol. Chem.*, **198,** 721 (1952).
14. Racker, E., and Krimsky, I., *J. Biol. Chem.*, **198,** 731 (1952).
15. Harting, J., and Velick, S., *Federation Proc.*, **11,** 226 (1952).
16. Wiggers, C. J., and Werle, J. M., *Proc. Soc. Exp. Biol. and Med.*, **49,** 604 (1942).
17. Baez, S., Zweifach, B. W., and Shorr, E., *Federation Proc.*, **11,** 7, 177 (1952).
18. Wiggers, H. C., Goldberg, H., Roemhild, F., and Ingraham, R. C., *Circulation*, **2,** 179 (1950).
19. Granick, S., *Federation Proc.*, **13,** 219 (1954).
20. Tecce, Di G., and Tecce, T., *Quad. nutr.*, **11,** 42 (1950).
21. Vosburgh, G. J., and Flexner, L. B., *Am. J. Physiol.*, **161,** 202 (1950).
22. Mazur, A., and Shorr, E., *J. Biol. Chem.*, **182,** 607 (1950).
23. Farrant, J. L., *Biochim. et biophys. acta.*, **13,** 569 (1954).
24. Starkenstein, E., and Weden, H., *Arch. exp. Path. u. Pharmakol.*, **134,** 274 (1928).
25. Barkan, G., *Z. physiol. Chem.*, **216,** 1, 17 (1933); **221,** 241 (1933).
26. Tompsett, S. L., *Biochem. J.*, **28,** 1536, 1544, 1802 (1934); **34,** 959, 961 (1940).
27. Laurell, C.-B., *Acta physiol. Scand.*, **14,** suppl. 46, 1 (1947).
28. Walker, J., and Turnbull, E. P. N., *Lancet*, **2,** 312 (1953).

KINETIC STUDIES ON THE PHENOL-SULFATE CONJUGATING SYSTEM OF RAT LIVER*

By HAROLD L. SEGAL†

(From the Department of Zoology, University of California, Los Angeles, California)

(Received for publication, August 2, 1954)

Conjugation of hydroxyl groups with sulfate and glucuronate *in vitro* has been accomplished with whole cell preparations (1–4) and homogenates (5–8). In this paper experiments are presented which demonstrate that the conjugation is a two-step reaction in which an activated form of sulfate is an intermediate, and that both reactions are sulfhydryl-dependent.

The present study has been confined to conjugation with sulfate and was undertaken in order to obtain information pertaining to the mechanism of the reaction.

A preliminary report has been presented (9).

EXPERIMENTAL

Enzyme System—The preparation of the enzyme system was based on the method of Bernstein and McGilvery (8). Adult, male, Sprague-Dawley rats were decapitated and the livers placed in ice-cold isotonic NaCl. 2 to 3 gm. portions were homogenized in 4 volumes of ice-cold isotonic KCl containing 10^{-3} M neutralized Versene and centrifuged at 18,000 × *g* for 2 hours at 0°. The supernatant fraction contained the phenol-sulfate conjugating activity. For most of the experiments described herein a further purification step was performed. To the supernatant fluid in the cold was added saturated ammonium sulfate containing 10^{-3} M Versene to a concentration of 1.7 M ammonium sulfate. The precipitated protein was centrifuged and discarded. The concentration of ammonium sulfate was raised to 2.3 M; the precipitated protein centrifuged, dissolved in one-third the original volume of KCl-Versene, and frozen in 1 ml. portions at −20° for future use. No decrease in activity was noted for at least 3 weeks under these conditions. Concentrations of protein were determined by Kjeldahl nitrogen × 6.25.

* This investigation was supported by a research grant to Dr. C. M. Szego from the National Cancer Institute of the National Institutes of Health, United States Public Health Service (C-1488).

† Present address, Department of Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania.

m-Aminophenol—The Eastman product was twice recrystallized from hot water with charcoal.

ATP[1]—The disodium salt of the Pabst product was used.

Method of Assay

The degree of conjugation was determined by the Bratton-Marshall method (10), in which the amino group is diazotized and coupled with NEDA, giving rise to a red compound which can be determined colorimetrically at 555 mμ (Fig. 1). By carrying out the color reaction under these

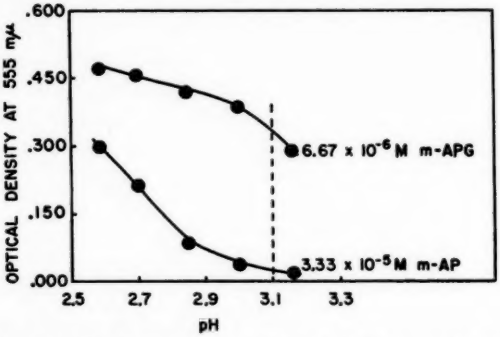


FIG. 1. Optical density given by free *m*-AP and the glucuronide as a function of the pH of color development. To 3.0 ml. of chromogen in 0.33 M citrate buffer was added 1.0 ml. of 0.05 per cent $NaNO_2$, followed in 3 minutes by 1.0 ml. of 0.5 per cent $NH_4SO_3NH_2$, followed in 4 minutes by 1.0 ml. of 0.1 per cent NEDA. The color was developed for 15 minutes. The entire reaction was carried out at 25°. The concentrations of *m*-AP and *m*-APG referred to are final. Optical density was measured in a Coleman junior spectrophotometer in 19 mm. tubes. The pH was measured at 25° before addition of nitrite, etc.

conditions at pH 3.1, the amount of conjugate could readily be measured, with very little color in the blanks from unconjugated phenol. *m*-APG was used as a standard, which according to Bernstein and McGilvery (8) is chromogenically equivalent to *m*-APS. The *m*-APG was a gift from Professor R. T. Williams. Delaying the addition of the NEDA for 4 minutes after the addition of the ammonium sulfamate, so as to allow more complete discharge of the nitrite, increased the final color and decreased its dependence on the time of addition of NEDA. Temperature, as well as pH and time of additions, is critical if reproducible results are to be obtained.

[1] The following abbreviations are used: ATP = adenosinetriphosphate; NEDA = *N*-naphthylethylenediamine; *m*-AP, *m*-APS, and *m*-APG = *m*-aminophenol, the sulfate, and the glucuronide, respectively; IAA = iodoacetic acid.

Aliquots of 0.3 to 0.6 ml. of the reacting system were removed at suitable times and added to 1.0 ml. of 1.0 M citrate plus sufficient water to make a final volume of 3.0 ml. and a final pH of 3.1. When the aliquots were added to the citrate at 0°, the reaction stopped immediately and no interfering protein turbidity was encountered. Similar aliquots from an identical incubation mixture kept at 0° served as blanks. No change in optical density of the blanks occurred when aliquots were taken over a 2 hour period from the same reaction medium at 0°.

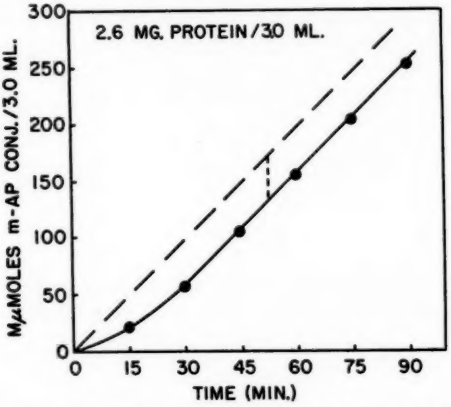


FIG. 2. Time-course of conjugation reaction. 3.0 ml. of medium contained 1.5 ml. of a solution of 0.3 M potassium phosphate buffer, pH 7.0, 0.03 M K_2SO_4, 0.005 M $MgCl_2$, and 0.002 M *m*-AP, plus 0.15 ml. of 0.08 M Na_2ATP and enzyme plus KCl-Versene to final volume. The reaction was started by addition of the enzyme. The temperature was 37° and the final pH 6.6. The dash line was constructed from the origin parallel to the linear part of the experimental curve. The vertical dotted line is the distance between the curves.

Results

Kinetics—The course of the reaction, with its usual lag period, can be seen from the typical curve in Fig. 2 (solid line). It was found that the kinetics of a two-step reaction fit such a curve and allow calculation of the rates of the steps individually.

The general reaction may be written

$$A + B + \cdots \xrightarrow[k_1]{} I \xrightarrow[k_2]{C + D + \cdots} P$$

where reactants A, B, etc., are pictured as giving rise to an intermediate I, which then reacts with C, D, etc., to form products, P. The rate constants, k_1 and k_2, contain the enzyme concentration. Under the experi-

mental conditions none of the reactants was rate-limiting. Therefore, the velocity of product formation is

$$\frac{dP}{dt} = k_2 [I] \tag{1}$$

The concentration of I is considered to be small at all times, and therefore the rate may be assumed to be approximately first order in $[I]$.

At steady state, that is on the linear portion of the experimental curve (Fig. 2), the velocity becomes equal to k_1, since at steady state

$$\frac{dI}{dt_{\text{st.st.}}} = k_1 - k_2 [I]_{\text{st.st.}} = 0$$

Therefore,

$$k_1 = k_2 [I]_{\text{st.st.}} \tag{2}$$

Therefore,

$$\frac{dP}{dt_{\text{st.st.}}} = k_1 \tag{3}$$

Therefore, the slope of the linear part of the curve gives k_1.

Next the equation of the experimental curve is obtained.

$$\frac{dI}{dt} = k_1 - k_2 [I]$$

Integrating from 0 to I and from 0 to t,

$$I = \frac{k_1}{k_2} (1 - e^{-k_2 t}) \tag{4}$$

From Equations 1 and 4

$$\frac{dP}{dt} = k_1(1 - e^{-k_2 t}) \tag{5}$$

the velocity at any time.

Integrating from 0 to P and from 0 to t,

$$P = k_1 t - \frac{k_1}{k_2} (1 - e^{-k_2 t}) \tag{6}$$

the amount of product at any time.

From Equation 5 or 6 it can be seen that the magnitude of k_2 determines the extent of the lag period, while only k_1 determines the final rate. When k_2 or t is sufficiently large so that $e^{-k_2 t}$ approaches 0 (is insignificant with respect to 1), the curve becomes linear.

The broken line (Fig. 2) is drawn parallel to the linear portion of the

experimental curve, and its equation is, therefore, $P = k_1 t$. The distance between the two lines must then be the second term on the right-hand side of Equation 6; *i.e.*, $k_1/k_2(1 - e^{-k_2 t})$. This value increases with time until $e^{-k_2 t}$ becomes negligible (*i.e.*, at steady state), when the distance between the lines becomes constant and equal to k_1/k_2. Since k_1 can be obtained from the slope (Equation 3), k_2 can then also be calculated. From Equation 2 it can be seen that the vertical distance between these lines is also equal to $[I]_{st.st.}$, since $[I]_{st.st.} = k_1/k_2$.

Effect of Enzyme Concentration—In Figs. 3 and 4 are plotted the values of the rate constants, as calculated by the above method, at different enzyme concentrations. It is apparent that the rates, when calculated in this

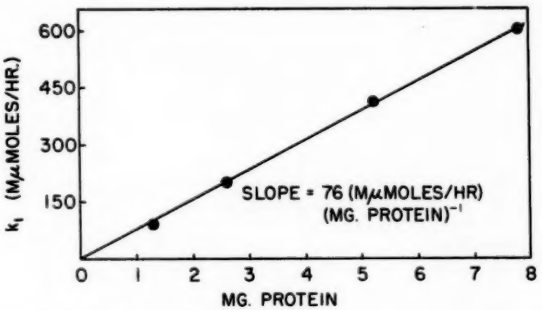


FIG. 3. Dependence of k_1 on enzyme concentration. Conditions as in Fig. 2

manner, are proportional to the enzyme concentration. The slopes of the lines give the rate constants per mg. of enzyme protein.

$[I]_{st.st.}$ is equal to the ratio of the rate constants at a given protein concentration (see above). Therefore, the ratio of the slopes in Figs. 3 and 4 gives an average value of $[I]_{st.st.}$ of 13×10^{-6} M/1.

Sulfate Activation—When the otherwise complete system was preincubated without the *m*-AP substrate and the reaction started by the addition of *m*-AP, the initial velocity was *greater* than at steady state, instead of smaller, but the same rate was approached as in a control (Fig. 5) preincubated in the absence of ATP.

Bernstein and McGilvery (11) have interpreted similar observations as an indication that the sulfate is activated as a first step in the over-all reaction, followed by coupling with the phenol. From Fig. 5 it can be inferred that, with preincubation, the intermediate *I*, which is presumed to be activated sulfate, was built up to a higher than steady state level, and hence the initial velocity was more rapid than steady state velocity until the concentration of intermediate fell to its steady state level.

Effect of Inhibitors—By means of the above kinetic analysis the effect of inhibitors on the individual steps can be determined.

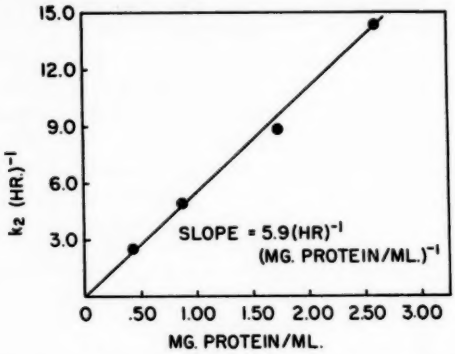


FIG. 4. Dependence of k_2 on enzyme concentration. Conditions as in Fig. 2

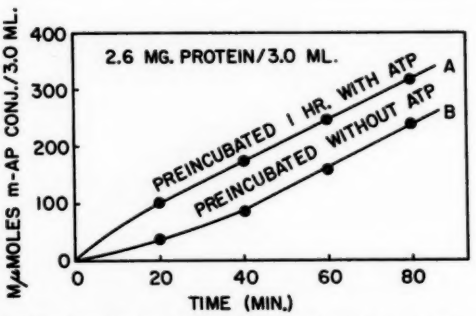


FIG. 5. Effect of preincubation in the absence of *m*-AP. 1.7 ml. of preincubation Mixture A contained 0.9 ml. of 0.52 M potassium phosphate buffer, pH 7.0, 0.15 ml. of 0.3 M K_2SO_4, 0.3 ml. of 0.025 M $MgCl_2$, 0.2 ml. of enzyme solution containing 2.6 mg. of protein, and 0.15 ml. of 0.08 M Na_2ATP. Preincubation Mixture B was identical, except no ATP was added. Preincubation for 1 hour at 37°. Reaction A was started by adding 1.7 ml. of preincubation Mixture A to 1.3 ml. of a solution containing 0.15 ml. of 0.02 M *m*-AP and 1.15 ml. of KCl-Versene. Reaction B was started by adding 1.55 ml. of preincubation Mixture B to 1.45 ml. of a solution containing 0.15 ml. of 0.02 M *m*-AP, 0.15 ml. of 0.08 M Na_2ATP, and 1.15 ml. of KCl-Versene.

In the presence of excess ATP an inhibition was obtained which was entirely on k_1, the rate constant of the activation reaction (Fig. 6). k_1 was decreased with increasing ATP addition with a proportional decrease in $[I]_{st.st.}$ so that k_2 remained constant within experimental error. Lower concentrations of ATP than 0.004 M did not further increase the rate. The

concentration of $I_{st.st.}$ was obtained in these experiments by extrapolating the linear portion of the experimental curves to the ordinate. The absolute magnitude of the intercept then is equal to $[I]_{st.st.}$. This is equivalent to the method used in Fig. 2.

IAA, on the other hand, increased the lag period, k_2 decreasing by about 75 per cent under these conditions, while the steady state velocity was decreased by only about 50 per cent (Fig. 7). Thus both the activating and coupling systems contain essential sulfhydryl groups, although the latter was more sensitive to IAA.

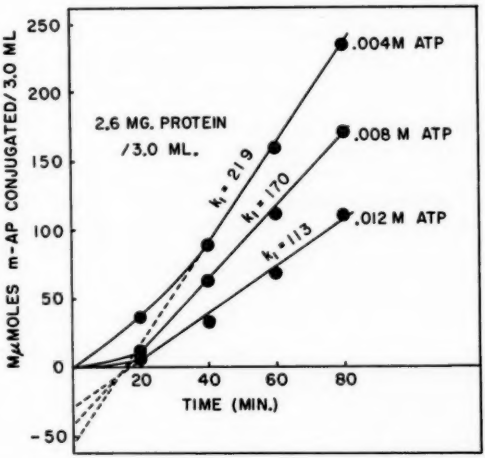


FIG. 6. Inhibition by additions of excess ATP. Conditions as in Fig. 2. Linear portions of the curves were extrapolated to zero time and the intercepts give $[I]$ as explained in the text. Units of k_1 as in Fig. 3.

The effects of substrates with respect to protection against IAA inhibition are summarized in Fig. 8. *m*-Aminophenol did not protect the enzyme. When the preincubation mixture contained ATP but no IAA, there was a slight increase in synthesis over the control containing no added ATP or IAA, for reasons already discussed (see Fig. 5). In the presence of ATP the enzyme was protected against IAA. In the absence of added Mg^{++} the protection was much reduced.

Other Substrates and Enzyme Sources—The system described above was also capable of conjugating estrone. After a 2 hour incubation in the presence of 5.8 γ per 3 ml. of estrone-16-C^{14} containing a total of 6420 c.p.m., essentially all of the radioactivity was converted to a form which was ether-insoluble, but which became ether-extractable after a 10 minute acid hy-

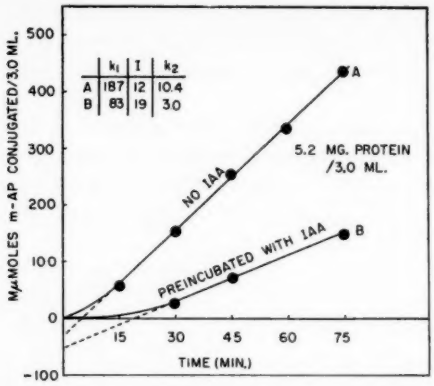


FIG. 7. Inhibition by IAA. Preincubations were for 1 hour at 37°. 1.8 ml. of preincubation mixtures contained 0.9 ml. of 0.52 M potassium phosphate buffer, pH 7.0, 0.3 ml. of 0.025 M $MgCl_2$, 0.15 ml. of 0.3 M K_2SO_4, 0.4 ml. of enzyme solution containing 5.2 mg. of protein, and 0.05 ml. of either 0.06 M IAA or H_2O. The reaction was started by addition of 1.8 ml. of the preincubation mixture to 1.2 ml. of a solution containing 0.15 ml. of 0.02 M *m*-AP, 0.15 ml. of 0.08 M Na_2ATP, 0.15 ml. of 0.1 M cysteine, and 0.75 ml. of H_2O. Units of k as in Figs. 3 and 4; I in micromoles per liter.

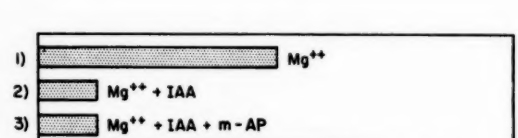


FIG. 8. Effect of substrates on IAA inhibition. Preincubation was for 1 hour at 37°. 2.1 ml. of preincubation mixtures contained 0.9 ml. of 0.52 M potassium phosphate buffer, pH 7.0, 0.15 ml. of 0.3 M K_2SO_4, 0.4 ml. of enzyme solution containing 5.2 mg. of protein, and, where indicated in Experiments 1 to 6, 0.3 ml. of 0.025 M $MgCl_2$, 0.05 ml. of 0.06 M IAA, 0.15 ml. of 0.02 M *m*-AP, 0.15 ml. of 0.08 M Na_2ATP, plus H_2O to volume. The reaction was started by addition of 2.1 ml. of preincubation mixture to 0.9 ml. of a solution containing 0.15 ml. of 0.1 M cysteine, plus the components of the complete system which were not included in the preincubation tubes, plus H_2O to volume.

drolysis.[2] When 10^{-3} M *m*-AP was also added, the coupling of the estrone was reduced two-thirds. There is little doubt that the product was estrone sulfate.

No phenol-sulfate conjugating activity was found in the high speed supernatant fractions after 2 hours centrifugation at 18,000 × *g* of homogenates of spleen, heart, kidney, or hepatoma induced by dietary butter yellow. The conjugating activity of liver is found in this fraction.

DISCUSSION

From the results presented it can be concluded that the enzymatic coupling of phenols with inorganic sulfate is a two-step reaction. The first is an activation step in which a sulfate-containing intermediate is formed with the expenditure of a high energy phosphate bond from ATP. In the second, or coupling, step the sulfate group is transferred from the intermediate to the phenol to form a phenol sulfate ester. Attempts to obtain evidence for a cofactor for the reaction, which might be a component of the intermediate, were unsuccessful. Dialysis of the enzyme preparation against Versene or distilled water in the cold led to complete loss of activity, which was not restored by adding the dialysate, under conditions in which an undialyzed control enzyme solution lost only a small fraction of its activity. Addition of the dialysate or boiled liver extract to undialyzed enzyme produced no stimulation.

Studies of the effects of inhibitors on the individual steps led to the conclusion that excess ATP, or more likely a contaminant of the ATP solution, is inhibitory to the activation reaction for which ATP is a substrate, but not to the coupling reaction. No attempt was made to purify the ATP further. IAA inhibited both reactions, but not to the same degree. Protection of both activities, however, was afforded by ATP plus Mg^{++}. Since it is not ATP but the activated sulfate formed from it which is the substrate of the second reaction, it is suggested that it is the latter which prevents IAA inhibition of the coupling step, particularly since Mg^{++} was necessary to maintain full activity. *m*-AP, which is also a substrate of the coupling reaction, gave no protection.

By analogy with the observations of Lynen *et al.* (12), who found that IAA would inactivate free but not acetyl-bound coenzyme A, and with the observations of Segal and Boyer (13), who found that IAA would inactivate free but not glyceraldehyde-3-phosphate-bound triosephosphate dehydrogenase, it may be inferred that ATP in the presence of Mg^{++} reacts directly with a sulfhydryl group of the activation system and that the active intermediate, or some part of it, reacts directly with a sulfhydryl group of the coupling system.

[2] Estrone-16-C^{14} was a gift to Dr. C. M. Szego from Professor R. D. H. Heard, McGill University.

SUMMARY

1. Rate curves of the phenol-sulfate conjugating system of rat liver have been shown to conform to the kinetics of a two-step reaction. The rate constants of the separate reactions have been determined by a graphical method. The rate constants, when calculated in this manner, are proportional to enzyme concentration. An activated form of sulfate is apparently an intermediate of the reaction.

2. Addition of increasing amounts of ATP caused an inhibition of the first, or activation, step but had no effect on the second, or coupling, reaction.

3. Iodoacetate inhibited the rates of both reactions. The inhibition was prevented by the presence of ATP plus Mg^{++} but not *m*-aminophenol.

4. Incubation of the system with estrone-16-C^{14} as a substrate gave rise to a product which was apparently estrone sulfate. No conjugating activity was found among the soluble enzymes of spleen, heart, kidney, or butter yellow-induced hepatoma, in contrast to the case with liver.

Thanks are due to Dr. C. M. Szego for the use of the facilities of her laboratories and to Miss D. Wolcott for performing the nitrogen analyses.

BIBLIOGRAPHY

1. Lipschitz, W. L., and Bueding, E., *J. Biol. Chem.*, **129,** 333 (1939).
2. De Meio, R. H., and Arnolt, R. I., *J. Biol. Chem.*, **156,** 577 (1944).
3. Levvy, G. A., and Storey, I. D. E., *Biochem. J.*, **44,** 295 (1949).
4. Storey, I. D. E., *Biochem. J.*, **47,** 212 (1950).
5. De Meio, R. H., and Tkacz, L., *Arch. Biochem.*, **27,** 242 (1950).
6. Dutton, G. J., and Storey, I. D. E., *Biochem. J.*, **48,** p. xxix (1951).
7. De Meio, R. H., and Tkacz, L., *J. Biol. Chem.*, **195,** 175 (1952).
8. Bernstein, S., and McGilvery, R. W., *J. Biol. Chem.*, **198,** 195 (1952).
9. Segal, H. L., and Szego, C. M., *Federation Proc.*, **13,** 132 (1954).
10. Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128,** 537 (1939).
11. Bernstein, S., and McGilvery, R. W., *J. Biol. Chem.*, **199,** 745 (1952).
12. Lynen, F., Reichert, E., and Ruoff, F., *Ann. Chem.*, **574,** 1 (1952).
13. Segal, H. L., and Boyer, P. D., *J. Biol. Chem.*, **204,** 265 (1953).

XUM

CORTICOTROPINS (ACTH)

I. ISOLATION OF α-CORTICOTROPIN FROM SHEEP PITUITARY GLANDS

By CHOH HAO LI, IRVING I. GESCHWIND, JONATHAN S. DIXON, ANTHONY L. LEVY,* AND J. IEUAN HARRIS

(From the Hormone Research Laboratory, University of California, Berkeley, California)

(Received for publication, August 9, 1954)

Since the discovery (1) of an adrenal-stimulating activity in the anterior pituitary and the subsequent demonstration (2) of the therapeutic value in rheumatoid arthritis of adrenocorticotropic concentrates (corticotropins) from pituitary glands, many attempts have been made to isolate this active principle from hypophyseal extracts (3, 4). A preliminary report has been made from this laboratory (5) of the isolation from *sheep* pituitary glands of a polypeptide possessing adrenocorticotropic (ACTH) activity. Since the composition of this hormone and many of its physicochemical characteristics differ from those reported by White (6) for corticotropin A isolated from hog glands, it has been designated α-corticotropin. In this and succeeding papers, we will report on its preparation, properties, and structure.

Isolation Procedure

Starting Material—The ACTH Fraction E obtained from the acid-acetone extract (AAP) of whole sheep pituitaries after adsorption on oxycellulose, as described previously (3, 7), was employed as the starting material for this work (Table I). This concentrate possesses approximately 25 U. S. P. units[1] of adrenal-stimulating activity per mg.

Dioxane Fractionation—Eastman dioxane was refluxed over sodium and distilled before use. 200 mg. of Fraction E were dissolved in 200 ml. of 50 per cent aqueous dioxane, and the solution was adjusted to pH 9.3 to 9.4 with 0.1 M NaOH. A flocculent precipitate which formed was removed by centrifugation; the clear supernatant fluid was immediately frozen and lyophilized. The resulting white powdered material (Fraction F) weighed about 100 mg. and contained ACTH activity equivalent to 45 U. S. P. units per mg., whereas the precipitate from dioxane contained less than 1 unit per mg. Measurements of pH were made with a glass electrode in a Leeds and Northrup pH meter.

* Deceased, August 22, 1954.

[1] All ACTH activities reported herein were estimated by comparing the effects of the unknown preparation in depleting ascorbic acid in the adrenals of hypophysectomized rats (8, 9) with those of the U. S. P. standard preparation.

Zone Electrophoretic Separation—Fraction F was next subjected to fractionation by means of zone electrophoresis on starch in a cold room at 3°. The technique employed was a modification of the method of Kunkel and Slater (10). Details, such as the type of trough used for the immobilized medium and the general procedure for packing the trough, were the same as those described previously (11). 100 mg. of Fraction F were dissolved in 1 ml. of 0.1 M Na_2CO_3 and placed on the trough about 6 cm. from the cathode end. The electrophoretic experiment was conducted at 200 volts for 16 hours. The starch in the trough was then cut into segments of 1 cm. and extracted three times with 5 ml. of water; a 0.1 ml. extract from each segment was analyzed for protein concentration by the method of Lowry

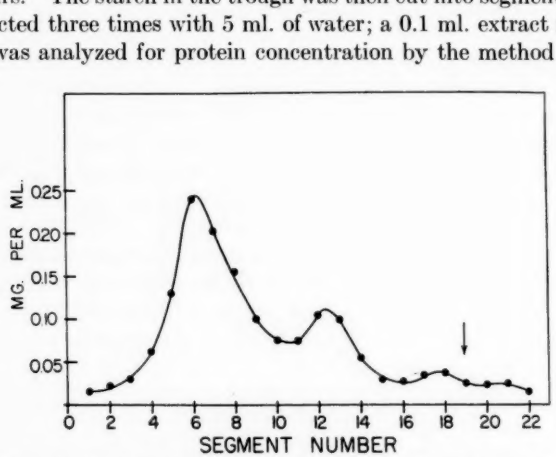


FIG. 1. Zone electrophoresis of Fraction F (see Table I) on starch; 40 cm. trough, 1 cm. per segment. pH 11.1 (0.1 M Na_2CO_3), 200 volts, 16 hours. The hormonal activity was concentrated in Segments 5 to 7.

et al. (12). A typical pattern may be seen in Fig. 1. Results of bioassay revealed that the active material was concentrated in Segments 5 to 7. The extracts from these segments were combined, dialyzed against distilled water for 5 to 6 hours at 0°, and lyophilized. The yield was 50 mg. of material (Fraction G) having an activity of 75 units per mg.

Chromatographic Fractionation on Ion Exchange Resin—Further purification of Fraction G was carried out by means of column chromatography, with the carboxyl cation exchange resin, Amberlite IRC-50, as adsorbent. The resin, in 250 to 500 mesh range (XE-97), was washed three times with 3 volumes of water; 10 minutes were allowed for settling after each washing. The resin was then prepared by a procedure similar to that described by Hirs *et al.* (13). After the resin had become converted completely to the sodium form, it was washed thoroughly with water, dried in a Büchner funnel, and stored in this form.

For preparation of a column, 250 ml. of the moist resin were stirred with 0.05 M $NaHCO_3$ for 1 hour and allowed to settle. The supernatant fluid was decanted, and sufficient 0.05 M $NaHCO_3$ solution was added to make a slurry, which was then poured into a column (2.4 × 25 cm.) fitted at the bottom with a coarse sintered glass disk. An additional 250 ml. of 0.05 M $NaHCO_3$ were allowed to flow through the column before Fraction G was applied.[2]

100 mg. of Fraction G in 25 ml. of 0.05 M $NaHCO_3$ were put onto the column; the column was then washed with 200 ml. of 0.05 M $NaHCO_3$, and the emergent solution was collected in 10 ml. fractions in a drop-counting fraction collector. After the 0.05 M $NaHCO_3$ solution had passed through the column, a gradual increase in the strength of the eluent was effected by allowing 350 ml. of 0.15 M $NaHCO_3$ to drop into 250 ml. of 0.05 M $NaHCO_3$ in the magnetically stirred reservoir in which mixing took place, as described previously ((14), *cf.* (15, 16)). 10 ml. fractions were collected in test-tubes, each of which contained 0.1 ml. of concentrated HCl. Finally, 300 ml. of 0.1 M Na_2CO_3 were passed through the column to elute the remainder of the adsorbed material. The concentration of material in each effluent fraction was estimated by measuring its absorption at 275 mμ in a model DU Beckman spectrophotometer; the result of a typical experiment may be seen in Fig. 2. Bioassay revealed that Peak II, containing 50 to 60 per cent of the original material, possessed the major part of the ACTH activity.

In order to accomplish the recovery of the active material, the contents of tubes 34 to 49 were combined, adjusted to about pH 4 with 1.0 M HCl, and lyophilized. In order to remove the salt, the dry solid (2 gm.) was dissolved in 50 ml. of water, and an equal volume of 50 per cent trichloroacetic acid (TCA) was added. The solution was then centrifuged, and the resulting supernatant fluid was discarded. The precipitate was dissolved in about 10 ml. of water, to which a few drops of 1.0 M HCl were added, and then extracted six times with an equal volume of peroxide-free ether. After careful removal under vacuum of the last traces of ether, the solution was lyophilized. The product (Fraction H) had an activity of 100 units per mg.; the yield was 50 mg.

Counter-Current Distribution—The final purification of α-corticotropin was achieved by counter-current distribution in a 2-butanol-aqueous trichloroacetic acid system. An automatic all-glass apparatus (17) consisting of 240 cells was employed, each cell in the train having been constructed to contain 5 ml. of lower, or stationary, phase. The counter-current dis-

[2] By altering the diameter of the column, the scale of the experiment has been increased (3.6 × 25 cm.) in order to prepare larger amounts of material or decreased (0.9 × 35 cm.) for analytical studies.

tribution experiments were carried out in a room maintained at 19° ± 1°. The following description of the steps involved in carrying out a specific purification of Fraction H may be taken as representative of the general procedure.

Distilled water and dry 2-butanol (Shell Chemical Corporation, distilled from $SnCl_2$) were shaken together in a volume ratio of 1.155:1 for equilibration, and the two layers were separated. The lower phase was made up to 0.2 per cent trichloroacetic acid by the addition of a calculated quantity of 50 per cent (weight per volume) TCA solution, shaken with an equal

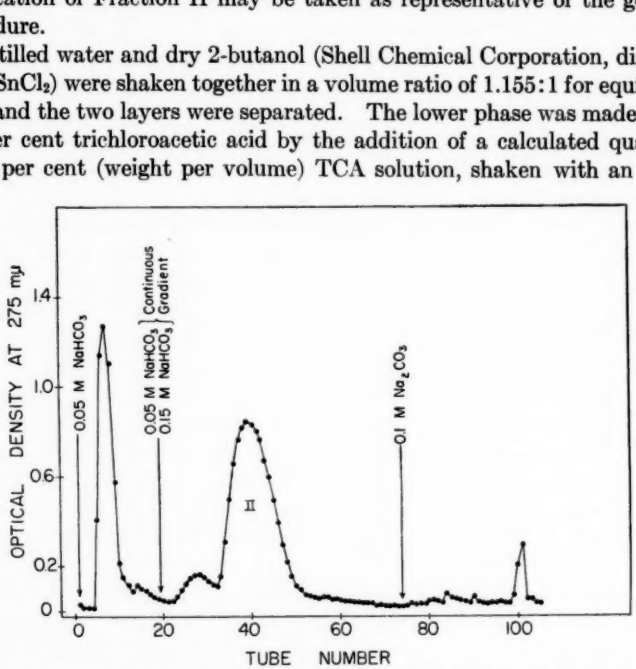


FIG. 2. Chromatography on the Na form of Amberlite IRC-50 resin (column 2.4 × 25 cm.) of material (Fraction G, 100 mg.) obtained from zone electrophoresis; 10 ml. per tube. The hormonal activity is located in tubes 34 to 49.

volume of upper phase, and allowed to equilibrate, with occasional shaking, for at least 24 hours before use.

The lower phase was then pipetted into each cell in the counter-current apparatus, and the device for the automatic addition of upper phase was put into operation. Preliminary experiments had shown that shaking the two layers for 70 seconds accomplished adequate mixing, while a settling time of 2.5 minutes was generally sufficient for good separation. The total time required for one complete transfer was 4.5 minutes.

After a forerun of twenty-four transfers had been completed, 236 mg. of Fraction H were dissolved in lower phase solvent, which had been removed from the 0 cell of the counter-current train, and returned to the cell along with 5 ml. of upper phase. This material was then submitted to a total

of 500 transfers, and the upper phase material issuing from the 239th (last) tube in the train was collected in an automatic fraction collector. After completion of the distribution, lower phase solution was removed for analysis. The optical density of each of these lower phase samples was measured at 275 mμ, with fresh lower phase as the reference blank, and the optical density of those upper phase samples which had been collected in the fraction collector was also measured, in this case against a water blank. No material was found in these upper phases. The experimental distribution of material as determined from the optical density measurements and the corresponding theoretical distribution are illustrated in Fig. 3.

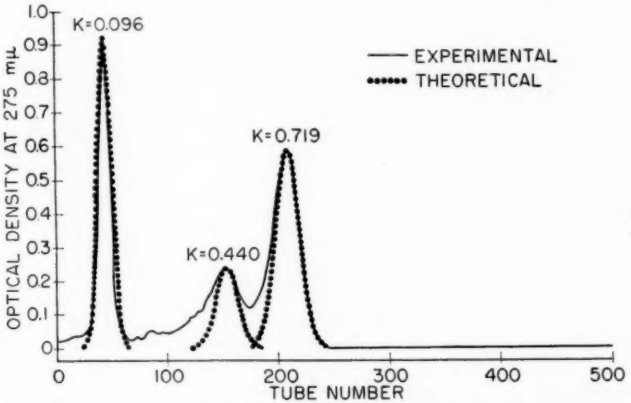


FIG. 3. Counter-current distribution (500 transfers) of material (236 mg. of Fraction H) obtained from chromatography on IRC-50 resin. System, 2-butanol-0.2 per cent aqueous trichloroacetic acid. The component with $K = 0.719$ has been designated α-corticotropin.

The material which had been distributed throughout the apparatus was combined into five fractions, designated Components *a*, *b*, *c*, *d*, and *e*, as follows: tubes 30 to 60, 80 to 135, 136 to 170, 171 to 203, and 204 to 239. The volume of each fraction was reduced to about 50 ml. at room temperature under reduced pressure; trichloroacetic acid was removed by extraction with ether, six successive extractions usually being sufficient to reduce the acidity of the water solution to pH 4.5 to 5. After extraction, each sample was taken to dryness *in vacuo* at room temperature, redissolved, and lyophilized for isolation of the material as the trichloroacetate salt. The yields were as follows: Component *a*, 46 mg.; Component *b*, 20 mg.; Component *c*, 32 mg.; Component *d*, 48 mg.; and Component *e*, 66 mg. Components *a* to *c* had an activity of less than 10 units per mg., whereas

Components *d* and *e* possessed equal potency equivalent to 150 units per mg.; the latter two were combined. The active product isolated in this manner was designated α-corticotropin (5). Table I summarizes the yield and estimated ACTH potency of fractions obtained from each step of the isolation procedure. It may be noted that 0.012 gm. of α-corticotropin was isolated from 1 kilo of whole sheep pituitaries. The over-all yield with respect to activity was about 15 per cent.

Homogeneity Studies

Counter-Current Distribution—Approximately 25 mg. of α-corticotropin were submitted to 50 transfers in a 2-butanol-0.1 per cent aqueous tri-

TABLE I

Yield and Potency of Various Fractions Obtained from Procedure for Isolation of α-Corticotropin

Fraction	Procedure	Yield from 1 kilo fresh sheep glands	Estimated ACTH potency
		gm.	*U. S. P. units per mg.*
AAP	Acid-acetone extract	25	0.5
D	NaCl precipitation	6.5	2
E	Oxycellulose adsorption	0.2	25
F	Dioxane fractionation	0.1	45
G	Zone electrophoresis on starch	0.05	75
H	IRC-50 resin column	0.025	100
α-Corticotropin	Counter-current distribution	0.012	150

chloroacetic acid system. The distribution patterns may be seen in Fig. 4; the experimental pattern appears to follow very closely the theoretical distribution curve for a partition coefficient (K) of 0.41. Moreover, the K values were found to be constant throughout the curve when distribution coefficients were calculated from the contents of adjacent pairs of tubes (18).

Partition Chromatography—The success which Porter achieved with the technique of partition chromatography in his studies on insulin (19), led us to explore the utility of this method for investigating the homogeneity of α-corticotropin. Hyflo Super-Cel (Johns-Manville) treated with silane (General Electric Company, Dri-Film), as described by Howard and Martin (20), was employed as the supporting medium. The solvent system was that used by Porter (19) for insulin and had the following composition: 9 ml. of 5 M sodium dihydrogen phosphate adjusted to pH 3.0 by the addition of phosphoric acid; 6.67 ml. of ethyl Cellosolve, 3.33 ml. of butyl Cello-

solve, and 15 ml. of water; the glycol ethers were redistilled in the presence of $SnCl_2$. The preparation and operation of the column followed exactly the details described by Porter (19).[3]

The distribution of α-corticotropin in this system is in favor of the organic phase, with a partition coefficient of 7.3. Fig. 5 presents the chromatographic pattern obtained when 3 mg. of the hormone were run on a 6 gm. column. The peptide concentration in the effluent was estimated by ultra-

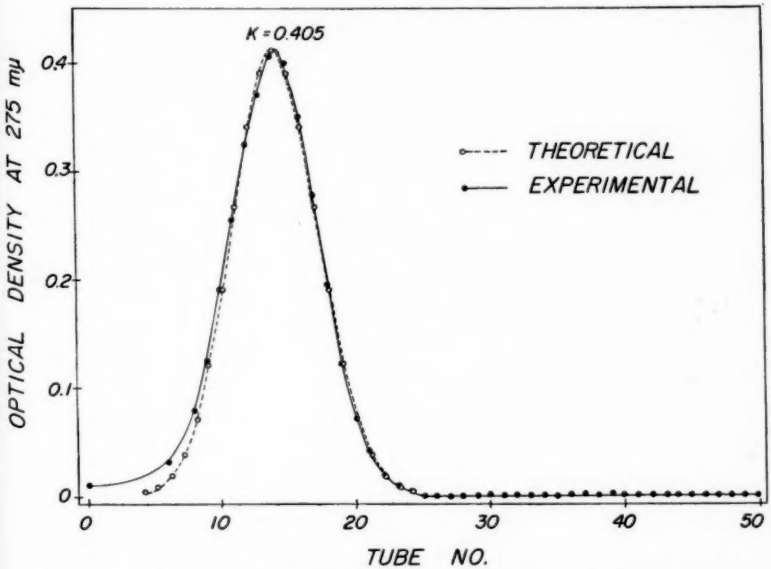


FIG. 4. Counter-current distribution (50 transfers) of α-corticotropin (see Fig. 3). System, 2-butanol-0.1 per cent aqueous trichloroacetic acid.

violet absorption at 275 mμ. α-Corticotropin behaved as a chromatographically homogeneous component ($R = 0.5$). When Fraction F was similarly chromatographed, its heterogeneity was indicated by at least five peaks on the effluent curve.

Zone Electrophoresis—The behavior of α-corticotropin in zone electrophoresis on starch at pH 5.1, 7.0, 8.2, 9.1, 10.2, and 11.2 has been examined, and in every case the hormone has been found to migrate as a single zone. The electrophoretic pattern obtained when 4 mg. of α-corticotropin were subjected to electrophoresis in an acetate buffer of pH 5.1 and 0.1 ionic

[3] The authors wish to thank Dr. R. R. Porter for the advice on these chromatographic experiments, which he so generously gave during his visit to this laboratory.

strength, at 170 volts for 16 hours, may be seen in Fig. 6. The experimental details were the same as those described above. Each segment along the curve appeared to possess equal activity according to biological assay.

FIG. 5. Chromatography of α-corticotropin (3 mg.). System, 15 ml. of water, 6.67 ml. of ethyl Cellosolve, 3.33 ml. of butyl Cellosolve, 9 ml. of 5 M NaH_2PO_4 (adjusted to pH 3 with H_3PO_4). Column, 6 gm. of silane-treated Hyflo Super-Cel.

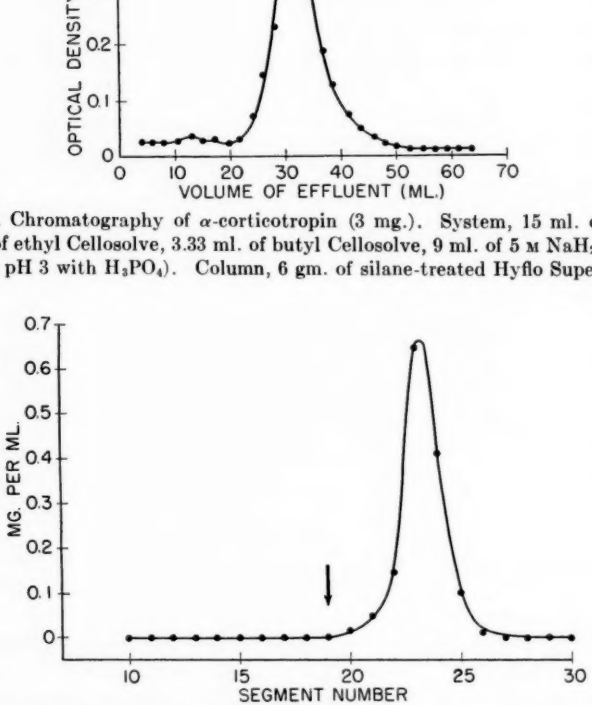


FIG. 6. Zone electrophoresis of α-corticotropin (4 mg.) on starch in acetate buffer of pH 5.1 and 0.1 ionic strength at 170 volts for 16 hours. The arrow indicates the site of application of the hormone.

Terminal Group and Amino Acid Analyses—Evidence for the purity of α-corticotropin has also been adduced on the basis of chemical studies. Briefly, reactions of α-corticotropin with phenyl isothiocyanate (21) revealed that serine occurs as the only *N*-terminal amino acid, in an amount close to 1 mole per mole (4541 gm.) of the peptide hormone (22). When

the hormone is treated with carboxypeptidase, 1 mole of phenylalanine is released per mole of α-corticotropin (22). The results of amino acid analyses of various preparations of α-corticotropin are in close agreement, and the molar ratios of the constituent amino acids very closely approximate whole numbers (23). These results suggest that the hormone peptide is a single chemical species.

Biological Tests—As has been mentioned above, α-corticotropin possesses an activity of 150 i.u. per mg. as estimated by the extent of ascorbic acid depletion in the adrenals of hypophysectomized rats. A daily dose of 0.005 mg. of α-corticotropin in beeswax-peanut oil suspension, injected into hypophysectomized rats on the day of operation and subsequently for 15 days, maintained normal adrenal weight at 23 mg., compared with 8 mg. for the controls. In experiments for measuring adrenal stimulation, a daily dose of 0.01 mg. in beeswax-peanut oil suspension given for 4 days to hypophysectomized male rats (operated on at 40 days of age and injected 4 days postoperatively) causes an increase of adrenal size from 9 to 24 mg. Moreover, a total dose of 1 mg. to hypophysectomized female rats (operated on at 27 days of age and injected for 4 days beginning on the 14th postoperative day) produces no histological evidence of the presence of thyrotropic and gonadotropic hormonal contaminations. Furthermore, a dose of 1 mg. injected locally into month-old squabs gives no indication of prolactational response. Bioassay for melanophore-expanding activity in hypophysectomized *Rana pipiens* indicates that α-corticotropin possesses an intermedin contamination of less than 0.1 per cent. A complete account of the biological behavior of the hormone will be reported elsewhere.

DISCUSSION

The successful concentration of ACTH activity has been achieved by a number of workers by means of ion exchange chromatography on oxycellulose (4, 24, 25) and on the polycarboxylic acid resin Amberlite IRC-50 (25–27). In this study, the use of gradually increasing cation concentration and pH on Amberlite IRC-50 (XE-97) has afforded an effective purification of α-corticotropin.

α-Corticotropin is adsorbed on IRC-50 at pH 8.3 if the sodium ion concentration is less than 0.10 M; at concentrations equal to or greater than 0.10 M the activity is no longer adsorbed. In actual practice it has been found expedient to introduce a sample, which has been purified by zone electrophoresis on starch, onto a column of resin equilibrated with 0.05 M $NaHCO_3$. Some inactive material passes through the column with the hold-up volume under these conditions, whereas the activity is strongly adsorbed. No further elution of material results even when quantities of buffer equal to as much as 20 hold-up volumes are passed through the

column. If a solution of 0.15 M $NaHCO_3$ is then put through the column, very rapid elution of activity occurs in the form of a single peak. All the remaining strongly adsorbed material can be removed by a final washing with a 0.1 M NaOH or 0.1 M Na_2CO_3 solution. The fraction[4] obtained with strongly alkaline solutions emerges with approximately 6 hold-up volumes of eluent and has an activity of less than 10 units per mg.

Since no special precautions had been taken to prevent contact of the bicarbonate solutions with air, with possible subsequent loss of CO_2 and change in pH, experiments were undertaken to demonstrate that elution of activity actually resulted from an increasing concentration gradient of sodium ions at constant pH. Mineral oil was layered on the bicarbonate solutions in order to prevent loss of CO_2. The pH of the effluent solutions was determined and found to be constant, and the distribution of ultraviolet-absorbing material was identical to that found in earlier experiments. Additional proof of the importance of the gradient of the sodium ion concentration was obtained by employing as eluent of the activity a solution which is 0.05 M with respect to $NaHCO_3$ and 0.10 M with respect to NaCl. The results showed that the distribution pattern of material was the same as that obtained with a 0.15 M $NaHCO_3$ solution. Thus, by simply increasing the sodium ion concentration at constant pH, elution of activity may be effected.

However, when a fraction derived from steps in the isolation procedure which *precede* the zone electrophoresis, such as Fraction F, is introduced onto the resin, chromatography on IRC-50 yields two incompletely resolved peaks which appear to be equally active (Fig. 7). Two minor components were also revealed; one of these appears just before the first active peak and is inactive, whereas the other follows the second major peak and possesses activity.

The results of a series of counter-current distributions in the system 2-BuOH-0.2 per cent TCA, performed on the active material (combined material of Peaks II_1 and II_2 in Fig. 7) may be seen in Fig. 8, *A*; the activity was confined to the peak having $K = 0.71$. The active peak was then cut and redistributed in the same system. The material giving the distribution pattern shown in Fig. 8, *B* was also cut and isolated. When each of these fractions was submitted to counter-current distribution, C_1, C_2, C_3, and C_4 were obtained. Chromatographic analyses indicate that C_4 contained both Peaks II_1 and II_2, whereas C_1 consisted almost exclusively of Peak II_2 when corrected for conversion taking place on the column (see below). Thus it appears that Peak II_1 has a slightly greater K value than Peak II_2 in this system, and that these two active components were not separated to a practical degree by the counter-current procedure.

[4] This fraction binds copper and travels down the column as a distinct blue band under the experimental conditions required for its displacement.

In order to clarify the relationship of these two active peaks and to ascertain why material obtained by zone electrophoresis on starch shows only Peak II_1, each of the two active peaks was rechromatographed on IRC-50 in the continuous gradient system. . While Peak II_1 behaved as a single component, chromatography of the second peak (II_2) revealed the presence of Peak II_1 as well as the expected Peak II_2; in fact, repeated chromatography of isolated Peak II_2 invariably led to the appearance of material in the Peak II_1 region.

It is apparent that Fraction F manifests two active peaks when chromatographed on a resin column; during the chromatography a gradual conversion

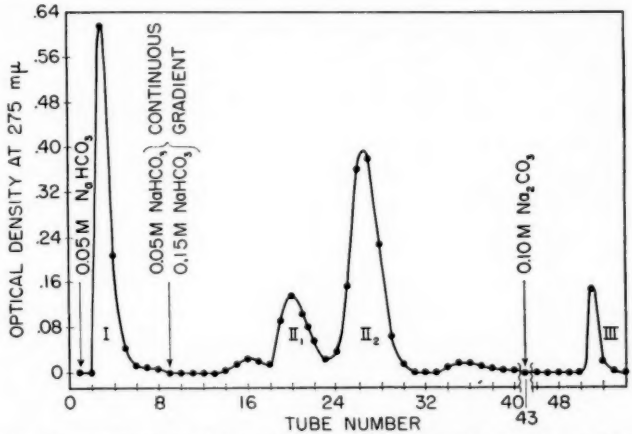


FIG. 7. Chromatography of Fraction F (100 mg.) on IRC-50 resin (column 2.3 × 26 cm.) with a continuous concentration gradient of $NaHCO_3$ solution.

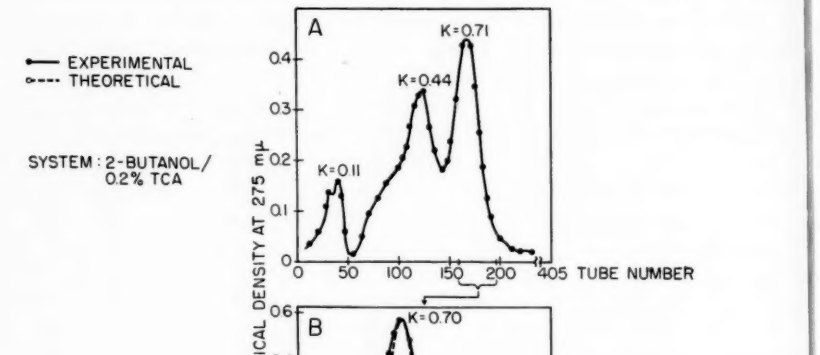
of Peak II_2 → Peak II_1 also occurs. This may mean that Peak II_1 represents at all times an artifact, created from Peak II_2 under the general conditions of isolation.[5]

Since *complete* conversion of Peak II_2 to Peak II_1 appears to take place during zone electrophoresis, it was thought probable that this conversion, like that which takes place on the columns, was due to the alkaline conditions maintained during the experimental procedure.[6] In fact, the extent

[5] It should be pointed out that there is no evidence of such a conversion occurring during the counter-current procedure, for, when Peak II_2 is submitted to counter-current analysis, followed by rechromatography, only as much Peak II_1 is found as is to be expected from the chromatography itself. It would follow, therefore, that the appearance of the two active peaks in the chromatographic experiments is not due to impurities present in Fraction F, since conversion on the column occurs even with the pure material isolated from the counter-current procedure.

[6] In most of the previous studies with IRC-50 phosphate buffers in the region of

of conversion was found to be dependent on pH as well as on time and temperature. Thus, more rapid and extensive conversion took place in 0.1 M Na_2CO_3 than in 0.2 M $NaHCO_3$, and complete conversion occurred if the starting material (Fraction F) was allowed to stand at room tempera-



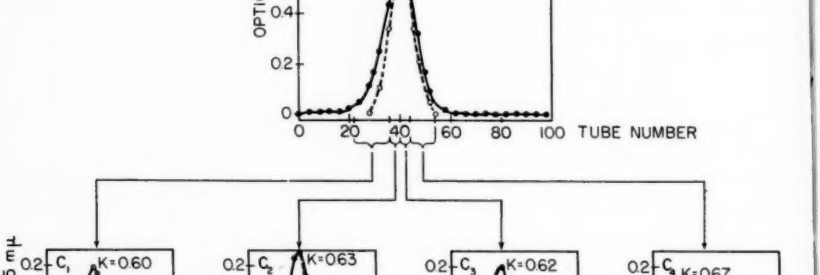


FIG. 8. Counter-current distribution of combined material of Peaks II_1 and II_2 (see Fig. 7). System, 2-butanol-0.2 per cent aqueous trichloroacetic acid.

ture for 16 hours in 0.1 M Na_2CO_3, whereas, after the same length of time at 6°, approximately 25 per cent of the total activity was still to be found in

pH 7 were employed (*e.g.* (13, 26)). The use of such neutral eluents is advantageous, because extremes of pH which may cause irreversible changes in proteins are avoided. It may be recalled, however, that the original application (28) of IRC-50 for the chromatography of a protein, cytochrome *c*, made use of buffers of pH 10.8. Similar alkaline conditions have been employed more recently for the chromatography of cytochrome *c* (29) and corticotropin A (27).

the second peak (II_2). After as short a period as 6 hours in 0.1 M Na_2CO_3 at room temperature, 90 per cent of the activity was found in Peak II_1.

On the basis of these results, it appears that conversion is undoubtedly due to the alkaline conditions under which zone electrophoresis on starch is performed. When the step involving electrophoresis is omitted, any conversion which occurs results not only from the alkaline conditions *per se* of the column, but also from the basicity of the solutions in the collecting tubes. To prevent conversion due to this latter cause, effluent was collected in tubes containing a small volume of concentrated HCl. Rechromatography of an active region which had been collected in this manner demonstrated that about one-half of the usual conversion had been prevented. Preliminary chemical investigations indicate that the hormone derived from Peak II_2 (α_2-corticotropin) possesses four amide groups per mole, whereas only two amide groups are found per mole of α-corticotropin. Since no value intermediate between two and four amide groups has been found, it would appear that two amide groups in α_2-corticotropin are probably alkali-labile. Moreover, qualitative carboxyl and amino terminal group analyses of these two fractions are the same. The finding of a greater K value for α-corticotropin than for α_2-corticotropin is consistent with similar findings with respect to insulin. Insulins A and B differ in their distribution characteristics in that Insulin B has the greater K value in the system 2-BuOH-0.1 per cent dichloroacetic acid (30), whereas they differ chemically only in that Insulin B possesses one amide group less than Insulin A (31).

It was pointed out earlier that counter-current distribution in a 2-butanol-aqueous TCA system is incapable of differentiating Peak II_2 from Peak II_1. On the other hand, the Peak II_1, derived from chromatography on the Amberlite IRC-50 (XE-97) column, can be easily resolved into at least three components, one of which possesses the partition coefficient of 0.72 in a 2-butanol-0.2 per cent TCA system (Fig. 3), and represents the active component (α-corticotropin). The K value for α-corticotropin in 2-butanol-aqueous TCA is sensitive to the concentration of TCA, whereas that of the inactive Component a varies very little. Fig. 9 presents the partition coefficient of α-corticotropin as a function of the concentration of TCA; a change in this concentration from 0.10 to 0.5 per cent causes an increase in K value from 0.42 to 3.2.

Although the results of this investigation demonstrate that counter-current distribution is indeed an effective tool for separation and isolation, it should be pointed out that caution must be employed in comparing experimental distribution curves with those plotted according to theory. The anomaly which appears in the experimental distribution curves presented in this investigation, namely, broadening of the distribution patterns, may

probably be explained in terms of a deviation from ideality of the partition isotherm which obtains for the particular peptide-solvent system under investigation (32). Experimentally, even a single pure component when fractionated in a non-ideal system may give a distribution curve which differs from what would be theoretically expected. In such cases the experimental distribution curve cannot be employed as the ultimate criterion for purity or lack thereof.

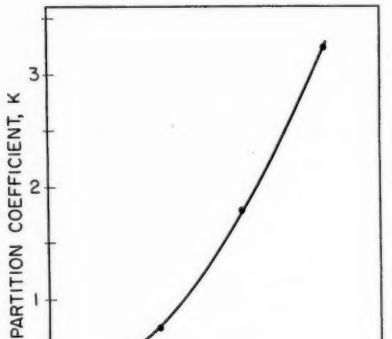


FIG. 9. Partition coefficient of α-corticotropin in 2-butanol-aqueous trichloroacetic acid system as a function of TCA concentration.

SUMMARY

A procedure involving dioxane fractionation, zone electrophoresis on starch, chromatography on Amberlite IRC-50 (XE-97) resin column, and counter-current distribution has been described for the isolation from sheep pituitary glands of a peptide which possesses ACTH activity equivalent to 150 i.u. per mg. The purity of this peptide, designated α-corticotropin, has been examined by means of counter-current distribution, partition chromatography, zone electrophoresis, terminal group and amino acid analysis, and biological tests.

Evidence is presented for the existence of an alkali-labile active precursor of α-corticotropin, whose behavior during the isolation procedure is discussed.

Grateful acknowledgment is made to Joan Elwood, Evelyn Emmrich, Elizabeth Klemperer, Winifred Lilly, Harold Papkoff, Ning G. Pon, and

Richard L. Wilcox, for their able technical assistance. This investigation was supported in part by grants from the National Institute of Health, United States Public Health Service (No. G-2907), and Eli Lilly and Company. We also wish to acknowledge the generous support of Merck and Company and the Armour Laboratories during the early stages of this investigation.

BIBLIOGRAPHY

1. Smith, P. E., *Am. J. Anat.*, **45,** 205 (1930).
2. Hench, P. S., Slocumb, C. H., Polley, H. F., and Kendall, E. C., *J. Am. Med. Assn.*, **144,** 1327 (1950).
3. Li, C. H., *Acta endocrinol.*, **10,** 255 (1952).
4. Astwood, E. B., Raben, M. S., and Payne, R. W., *Recent Progress Hormone Res.*, **7,** 1 (1952).
5. Li, C. H., Geschwind, I. I., Levy, A. L., Harris, J. I., Dixon, J. S., Pon, N. G., and Porath, J. O., *Nature*, **173,** 251 (1954).
6. White, W. F., *J. Am. Chem. Soc.*, **75,** 503 (1953).
7. Li, C. H., *J. Am. Chem. Soc.*, **74,** 2124 (1952).
8. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, **42,** 379 (1949).
9. Geschwind, I. I., Williams, B. S., and Li, C. H., *Acta endocrinol.*, **8,** 247 (1951).
10. Kunkel, H. G., and Slater, R. J., *Proc. Soc. Exp. Biol. and Med.*, **80,** 42 (1952).
11. Fønss-Bech, P., and Li, C. H., *J. Biol. Chem.*, **207,** 175 (1954).
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193,** 265 (1951).
13. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **200,** 493 (1953).
14. Clauser, H., and Li, C. H., *J. Am. Chem. Soc.*, **76,** 4337 (1954).
15. Donaldson, K. O., Tulane, V. J., and Marshall, L. M., *Anal. Chem.*, **24,** 185 (1952).
16. Busch, H., Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **196,** 717 (1952).
17. Craig, L. C., Hausmann, W., Ahrens, E. H., and Harfenist, E. J., *Anal. Chem.*, **23,** 1276 (1951).
18. Williamson, B., and Craig, L. C., *J. Biol. Chem.*, **168,** 687 (1947).
19. Porter, R. R., *Biochem. J.*, **53,** 320 (1953).
20. Howard, J. A., and Martin, A. J. P., *Biochem. J.*, **46,** 532 (1950).
21. Edman, P., *Acta chem. Scand.*, **4,** 283 (1950).
22. Harris, J. I., and Li, C. H., *J. Am. Chem. Soc.*, **76,** 3607 (1954).
23. Levy, A. L., Geschwind, I. I., and Li, C. H., *J. Biol. Chem.*, **213,** 187 (1955).
24. Geschwind, I. I., Porath, J. O., and Li, C. H., *J. Am. Chem. Soc.*, **74,** 2121 (1952).
25. Richter, J. W., Ayer, D. E., Bazemore, A. W., Brink, N. G., and Folkers, K., *J. Am. Chem. Soc.*, **75,** 1952 (1953).
26. Dixon, H. B. F., Moore, S., Stack-Dunne, M. P., and Young, F. G., *Nature*, **168,** 1044 (1951).
27. White, W. F., and Fierce, W. L., *J. Am. Chem. Soc.*, **75,** 245 (1953).
28. Paléus, S., and Neilands, J. B., *Acta chem. Scand.*, **4,** 1024 (1950).
29. Margoliash, E., *Biochem. J.*, **56,** 529 (1954).
30. Harfenist, E. J., and Craig, L. C., *J. Am. Chem. Soc.*, **74,** 3083 (1952).
31. Harfenist, E. J., *J. Am. Chem. Soc.*, **75,** 5528 (1953).
32. Craig, L. C., *Rec. Chem. Progress*, **14,** 177 (1953).

XUM

By

In
from
its t
amin
recen

Hy
13.3
0.5 n
remo
evap
ml. o
Qu
0.1 n
analy
butan
urate
pape
mato
curri
cysti
visibl
even
phot
by o
phth
Qu
was
amin
siona

* I
¹ T
note

CORTICOTROPINS (ACTH)

II. AMINO ACID COMPOSITION OF α-CORTICOTROPIN

By ANTHONY L. LEVY,* IRVING I. GESCHWIND, AND CHOH HAO LI

(From the Hormone Research Laboratory, University of California, Berkeley, California)

(Received for publication, August 9, 1954)

In Paper I of this series (1), a procedure was described for the isolation from sheep pituitary glands of a pure peptide, α-corticotropin (ACTH), as its trichloroacetate. The object of this communication[1] is to report the amino acid composition of this peptide hormone as determined by a method recently developed in this laboratory.

EXPERIMENTAL

Hydrolysis—α-Corticotropin trichloroacetate (2 to 5 mg., N content 13.3 per cent) was hydrolyzed at 110° in a sealed evacuated tube with 0.5 ml. of constant boiling (5.7 N) HCl which had been glass-distilled to remove trace metals (3). After hydrolysis for 24 hours, the acid was evaporated over a steam bath, and the dry residue was taken up with 3.0 ml. of CO_2-free water.

Qualitative Analysis—A known volume of the hydrolysate, derived from 0.1 mg. of α-corticotropin, was applied to a Whatman No. 52 paper for analysis by two-dimensional chromatography in the solvent system *n*-butanol-acetic acid-water (4:1:5), followed by *m*-cresol-phenol (1:1) saturated with borate buffer at pH 9.3, as described previously (4). The paper was sprayed with a solution of ninhydrin (4) to produce the chromatogram shown in Fig. 1. It will be seen that all of the commonly occurring amino acids are present in α-corticotropin, with the exception of cystine, threonine, and isoleucine. The spot due to histidine was clearly visible on the original chromatogram because of its turquoise-blue color, even though it cannot be distinguished from arginine on the black and white photocopy. The presence of leucine rather than isoleucine was confirmed by one-dimensional chromatography in the system tertiary amyl alcohol-phthalate at pH 6.

Quantitative Analysis—The amino acid composition of α-corticotropin was determined by converting the hydrolysate to *N*-dinitrophenyl (DNP)-amino acids and submitting these derivatives to quantitative two-dimensional chromatography on filter paper (5).

* Deceased, August 22, 1954.

[1] The results of this study have already been published in part, as a preliminary note (2).

In a typical experiment, the hydrolysate (3.0 ml.) of an α-corticotropin preparation (No. CC9HD, 4.55 mg., N content 13.3 per cent) was prepared as described above, 0.10 ml. of 3.1 N KCl was added, and the pH was adjusted to 9.00 by the addition of 40.0 μmoles of 0.20 N NaOH. The solu-

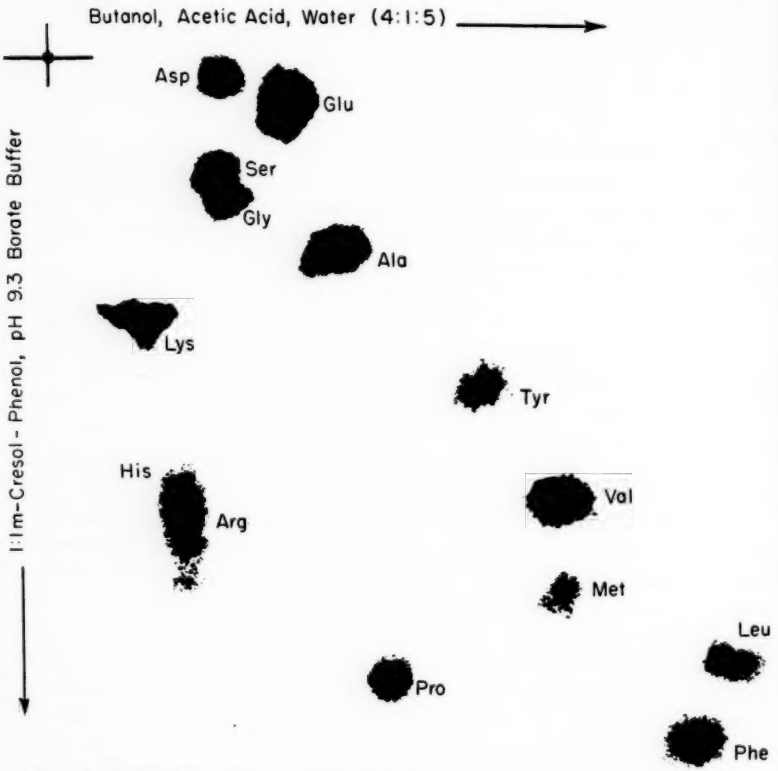


FIG. 1. A two-dimensional chromatogram on paper of a 24 hour hydrolysate of α-corticotropin trichloroacetate (5.7 N HCl; 110°).

tion was then saturated with 1,2,4-fluorodinitrobenzene (FDNB) at 40° by vigorous stirring with a slight excess (about 0.1 ml.) of the reagent, and the pH was maintained at 9.00 for 80 minutes by intermittent additions of 0.20 N NaOH. This operation was effected by means of the autotitrator described by Jacobsen and Léonis (6); the titrator record of alkali uptake as a function of time is shown in Fig. 2. Extrapolation of the linear end-slope, which resulted from the formation of dinitrophenol, to zero time,

showed that 49.0 μmoles of OH^- were consumed owing to reaction of FDNB with the amino acids of the hydrolysate.

The solution was then extracted with peroxide-free ether (twice with 5 ml.) to remove excess FDNB, acidified with 5 N hydrochloric acid (0.5 ml.), and the DNP-amino acids were extracted into ether (five times with 5 ml.). The aqueous solution which contained α-DNP-arginine and α-DNP-histidine was diluted to 10 ml. Three 2 ml. aliquots of the ether extract and three 1 ml. aliquots of the water solution were evaporated to dryness, and the yellow residues were transferred to adjacent corners of three sheets of 17 × 22.5 inch Whatman No. 1 filter paper with the aid of acetone (containing a few drops of concentrated HCl in the case of the water-soluble DNP-amino acids). The papers were then run overnight by the ascending procedure with the toluene-chloroethanol-pyridine-0.8 N ammonia system (modified to 5:3:1.5:3) of Biserte and Osteux (7). The chromatograms were dried for 3 to 4 hours at 40° and the spots due to α-DNP-arginine and α-DNP-histidine excised at this point. The papers were then run overnight in the second dimension by the descending procedure with 1.6 M phosphate buffer at pH 6 as irrigating fluid, to give the pattern of spots in Fig. 3.

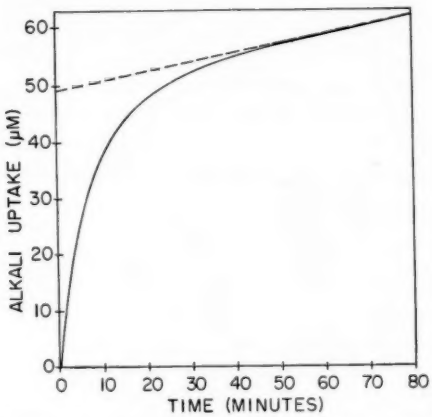


FIG. 2. Alkali uptake accompanying the reaction of a hydrolysate of α-corticotropin with FDNB at pH 9.0 and 40° (μ = 0.1 M KCl).

The spots were cut out and dropped into a set of labeled test-tubes; three pieces (3 × 4 cm.) of blank paper were also cut from each sheet in the position shown (Fig. 3). A dividing line between DNP-aspartic and DNP-glutamic acids was fairly clear when the papers were viewed under

ultraviolet light. In the case of DNP-methionine, the two spots marked "Met" and "ox. Met" were cut out and combined. 4 ml. of 1 per cent sodium bicarbonate were pipetted into each of the tubes, which were then placed in a water bath at 55 to 60° for 15 minutes to allow elution of the color. After an additional 15 minutes for the solutions to cool to room temperature, they were decanted successively into a 1 cm. quartz cuvette, and the optical densities at 360 mμ (385 mμ in the case of DNP-proline)

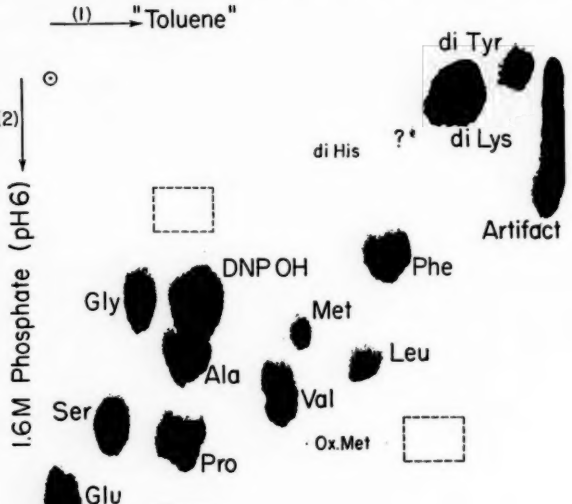


FIG. 3. A two-dimensional chromatogram on paper of a dinitrophenylated hydrolysate of α-corticotropin trichloroacetate. The dotted areas were cut to serve as paper blanks.

were read in the Beckman model DU spectrophotometer against a water blank. The mean optical densities of the tubes containing the blank papers were 0.017, 0.014, and 0.022 for the three chromatograms, and the appropriate corrections were made for each spot according to its estimated size.

In order to convert the optical densities to molar ratios, they were multiplied by the set of factors F (recorded previously (5)), which have been found empirically from the analysis of synthetic mixtures of amino acids. Since the absolute recoveries from the triplicate chromatograms were different,[2] the results were then expressed on a common basis as molar frac-

[2] The differences are usually somewhat greater than in the present experiment.

tions (see Table I), and the mean molar fractions for the three chromatograms were computed. The number of amino acid residues per minimal molecular weight were obtained directly (seventh column, Table I) by dividing the mean molar fractions by that number (0.0268) which gave a closest approximation to whole number ratios for the greatest number of amino acids. It was at once evident that most of the amino acid residues were in stoichiometric ratios and that α-corticotropin contained a minimum of thirty-seven amino acids, exclusive of losses due to destruction by the acid. No attempt was made to calculate recovery on the basis of weight (gm. of amino acid residue per 100 gm. of peptide), in view of the uncertainty concerning the trichloroacetic acid content of the preparation.

Three other preparations of α-corticotropin trichloroacetate were analyzed on widely different occasions by the paper-DNP technique described above. The molar ratios and other data from these analyses are collected in Table I; the values for serine are corrected (10 per cent) for destruction after 24 hours of hydrolysis (8). The fact that a non-integral value still results suggests that the correction factor for serine is somewhat too low in this case. With Preparation CC5HI, the separation of DNP-aspartic and DNP-glutamic acids was not complete, and these particular values therefore have not been included in the final mean; however, their sum is the same as the sum of these two DNP-amino acids according to the other analyses.

Determination of Tyrosine and Tryptophan—Since tryptophan is destroyed by acid hydrolysis, it was estimated from the ultraviolet light absorption of the intact peptide according to the procedure of Goodwin and Morton (9) and found to be present in an amount of 1.10 moles per mole of α-corticotropin. This method also allows an independent determination of the tyrosine content of α-corticotropin, which was 1.88 residues per mole, in good agreement with the value of 2.13 residues per mole obtained by the FDNB technique.

Determination of Amide Content—Ammonia was determined both by hydrolyzing the peptide (4 mg.) for 10 days at 37° with concentrated hydrochloric acid according to the method of Rees (8) and by means of a method[3] which is carried out without prior hydrolysis of the sample. According to the latter procedure, 4 mg. of the hormone were dissolved in 1.0 ml. of H_2O, and the solution was introduced into a Pregl-Parnas-Wagner distillation apparatus, such as that used for Kjeldahl distillation. After the addition of 10 ml. of 30 per cent NaOH and sufficient water to make a final volume of 30 ml., steam distillation was allowed to proceed for exactly 8 minutes. The ammonia formed was trapped in a boric acid solution and titrated with 0.01 N H_2SO_4 with bromcresol green-methyl red as indicator.

[3] Geschwind, I. I., unpublished work.

TABLE I

*Amino Acid Composition of α-Corticotropin**

Amino acid	Preparation CC9HD					Minimal residues per molecule					
	Results as molar fractions			Mean molar fraction	Per cent deviation from mean	Preparation No.				Mean ± deviation from mean	Residues to nearest integer
	Chromatogram No.					CC9HD	CC9HE	CC5HI	CC4I		
	I	II	III								
Aspartic	0.0392	0.0503	0.0491	0.0462	10.1	1.72	1.93	(2.63)	2.26	1.97 ±0.19	2
Glutamic	0.1345	0.1273	0.1282	0.1300	2.3	4.85	4.90	(4.15)	4.56	4.77 ±0.14	5
Serine	0.0689	0.0690	0.0702	0.0694	0.9	2.85†	2.77†	2.81†	2.98†	2.85 ±0.06	3
Glycine	0.0837	0.0818	0.0842	0.0832	1.2	3.10	3.05	2.84	3.04	3.01 ±0.08	3
Alanine	0.0747	0.0753	0.0753	0.0751	0.3	2.80	2.79	2.97	3.10	2.92 ±0.12	3
Proline	0.1107	0.1084	0.1087	0.1093	0.9	4.08	4.06	4.02	4.02	4.05 ±0.03	4
Valine	0.0798	0.0798	0.0816	0.0804	1.0	2.99	3.05	3.05	2.90	3.00 ±0.05	3
Methionine	0.0235	0.0211	0.0212	0.0219	4.7	0.82	0.76	0.73		0.77 ±0.03	1
Leucine	0.0291	0.0246	0.0268	0.0268	5.7	1.00	1.00	1.00	1.15	1.04 ±0.06	1
Phenyl-alanine	0.0822	0.0809	0.0823	0.0818	0.8	3.05	3.02	3.02	2.98	3.02 ±0.02	3
Tyrosine	0.0608	0.0647	0.0607	0.0621	2.8	2.32	1.92	2.07	2.19	2.13 ±0.13	2
Lysine	0.1049	0.1073	0.1018	0.1047	1.8	3.91	3.88	4.06	3.89	3.94 ±0.07	4
Histidine	0.0268	0.0272	0.0273	0.0271	2.1	1.01	1.03	0.87	0.96	0.97 ±0.05	1
Arginine	0.0810	0.0821	0.0825	0.0819	0.4	3.06	2.86	2.91	3.14	2.99 ±0.11	3
Tryptophan										1.10‡	1
Amide NH_3										2.08	2
Total.....	0.9998	0.9998	0.9999	0.9999	±2.5	37.6	37.0	37.1	37.2	40.6	41

* Sample of about 3 mg., hydrolyzed with constant boiling hydrochloric acid at 110° in a sealed tube.

† Corrected (10 per cent) for destruction.

‡ Determined on Preparation CC9HD by the spectrophotometric method of Goodwin and Morton (9).

The methods of both Rees (8) and Geschwind[3] afforded results which were in excellent agreement, giving values for the ratio of amide N to total N of 3.6 and 3.7 per cent, respectively. These values would allow 2.08 moles of ammonia per mole of α-corticotropin. The probable existence of four amide NH_3 groups in the naturally occurring precursor of α-corticotropin is discussed in Paper I (1).

TABLE II

*Amino Acid Nitrogen As Per Cent of Total Nitrogen in α-Corticotropin (Preparation CC9HD)**

Amino acid	Amount	N as per cent of total N
	μmoles	
Aspartic	1.32	3.06
Glutamic	3.74	8.66
Serine	1.99	4.62
Glycine	2.40	5.55
Alanine	2.16	5.01
Proline	3.14	7.28
Valine	2.30	5.32
Methionine	0.63	1.45
Leucine	0.77	1.79
Phenylalanine	2.34	5.42
Tyrosine	1.79	4.15
Lysine	3.01	13.93
Histidine	0.82	5.68
Arginine	2.25	20.84
Tryptophan		3.86
Amide NH_2		3.65
		100.3

* Analysis of 4.55 mg. of the peptide trichloroacetate (13.3 per cent N), corresponding to 0.605 mg. of total N; based upon the average of the three analyses summarized in Table I.

DISCUSSION

It will be seen that the mean values of the molar ratios in Table I are all very close to whole numbers, with the exception of those for glutamic acid, serine, and methionine. Destruction during hydrolysis (10–12) may be responsible for the low value of glutamic acid (4.77), and of methionine (0.77); the correction for the destruction of serine during acid hydrolysis has already been discussed. A kinetic study of the hydrolysis would be necessary to obtain the corrected values; in any case such investigations would not be expected to change the integral values of the residues per mole (last column of Table I). The consistency of the molar ratios of the

amino acids in four different preparations of α-corticotropin trichloroacetate, and their close approximation to whole numbers, is convincing evidence that a single chemical species has been isolated, the more so since this analysis together with the amide content accounts for the nitrogen of the peptide (Table II). The amino acid analysis indicates a minimal molecular weight of 4541 for α-corticotropin, which, in view of quantitative end-group studies (13), is probably the true molecular weight. The isoionic point of α-corticotropin is 9.3[4] when calculated from the amino acid

TABLE III

Comparison of Amino Acid Composition of Corticotropins

Amino acid	α-Corticotropin (sheep, this study)	Corticotropin A (hog, White (15))
Aspartic	2	1
Glutamic	5	4
Cystine	0	0
Serine	3	1
Threonine	0	0
Glycine	3	2
Alanine	3	2
Proline	4	3
Valine	3	2
Methionine	1	(1)*
Leucine	1	2
Isoleucine	0	0
Phenylalanine	3	2
Tryptophan	1	1
Tyrosine	2	1
Lysine	4	3
Histidine	1	1
Arginine	3	2
Amide NH_3	2	

* Personal communication from Dr. White.

and amide composition by the method of McLaren and Lewis (14). As a check, this method has yielded isoionic points within 0.05 pH unit of the values found experimentally for carboxypeptidase and papain, calculated from the composition recently reported for these two proteins (11, 12). The analysis gives a theoretical N content of 17.6 per cent for α-corticotropin; as isolated, α-corticotropin trichloroacetate has a nitrogen content of 13 to 14 per cent. No evidence for the optical form of the amino acids derived from α-corticotropin has been adduced.

[4] However, α-corticotropin trichloroacetate shows maximal insolubility at about pH 6.5, which is in good agreement with the value of 6.6 determined experimentally for its isoelectric point (Raacke, I. D., and Li, C. H., unpublished work).

It is of interest to compare the amino acid composition of α-corticotropin (isolated from sheep pituitaries) with the data reported for the amino acid composition of the porcine hormone, corticotropin A (15); a marked difference is evident in the molar ratios of the amino acids (see Table III). However, if the values for corticotropin A are multiplied by 3/2, the results parallel closely the molar ratios for α-corticotropin, with the exception of small differences in the leucine, glutamic acid, and serine content which might be ascribed to species specificity (see, for example, Harfenist and Craig (16) in connection with insulin); these latter differences may also explain the differences in the partition coefficients of the two peptides in the system 2-butanol-0.2 per cent aqueous trichloroacetic acid (1, 2, 15).

The paper-DNP method of amino acid analysis used in this investigation has followed the procedure described elsewhere (5) and has shown the expected accuracy. The method hitherto has been applied to the analysis of synthetic mixtures, insulin, and ribonuclease (5), and therefore this study represents the first application to the analysis of an entirely unknown substance.

The amide method reported here[3] has been used extensively in this laboratory for the past 5 years. With twelve different proteins whose amide contents are well documented, values within ±4 per cent of those reported in the literature have been obtained by this technique.

SUMMARY

The molar ratios of amino acids in α-corticotropin are as follows: alanine 3, ammonia 2, arginine 3, aspartic acid 2, glutamic acid 5, glycine 3, histidine 1, leucine 1, lysine 4, methionine 1, phenylalanine 3, proline 4, serine 3, tryptophan 1, tyrosine 2, and valine 3. These data were obtained by quantitative paper chromatography of the dinitrophenyl derivatives of the amino acids. The analysis indicates that α-corticotropin is composed of thirty-nine amino acids and 2 molecules of ammonia, with a calculated minimal molecular weight of 4541. The relationship of α-corticotropin to corticotropin A is discussed.

This investigation has been supported in part by grants from the National Institutes of Health, United States Public Health Service (No. G-2907), and Eli Lilly and Company, for which sincere appreciation is expressed. The authors wish to acknowledge the able assistance of Mr. David Chung during the course of this work.

Addendum—Since this paper was submitted for publication, a report by Bell (17) has appeared, describing the purification and structure of β-corticotropin derived from hog pituitaries. The amino acid composition of this peptide is strikingly similar to that reported here for sheep ACTH. β-Corticotropin apparently consists of one less serine and one more leucine than α-corticotropin; in addition, the porcine hormone has only one amide NH_3 group.

BIBLIOGRAPHY

1. Li, C. H., Geschwind, I. I., Dixon, J. S., Levy, A. L., and Harris, J. I., *J. Biol. Chem.*, **213,** 171 (1955).
2. Li, C. H., Geschwind, I. I., Levy, A. L., Harris, J. I., Dixon, J. S., Pon, N. G., and Porath, J. O., *Nature*, **173,** 251 (1954).
3. Jacobsen, C. F., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **26,** 463 (1949).
4. Levy, A. L., and Chung, D., *Anal. Chem.*, **25,** 396 (1953).
5. Levy, A. L., *Nature*, **174,** 126 (1954).
6. Jacobsen, C. F., and Léonis, J., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **27,** 333 (1951).
7. Biserte, G., and Osteux, R., *Bull. Soc. chim. biol.*, **33,** 50 (1951).
8. Rees, M. W., *Biochem. J.*, **40,** 632 (1946).
9. Goodwin, T. W., and Morton, R. A., *Biochem. J.*, **40,** 628 (1946).
10. Harfenist, E. J., *J. Am. Chem. Soc.*, **75,** 5528 (1953).
11. Smith, E. L., and Stockell, A., *J. Biol. Chem.*, **207,** 501 (1954).
12. Smith, E. L., Stockell, A., and Kimmel, J. R., *J. Biol. Chem.*, **207,** 551 (1954).
13. Harris, J. I., and Li, C. H., *J. Am. Chem. Soc.*, **76,** 3607 (1954).
14. McLaren, A. D., and Lewis, C., *J. Polymer Sc.*, **5,** 379 (1950).
15. White, W. F., *J. Am. Chem. Soc.*, **75,** 503 (1953).
16. Harfenist, E. J., and Craig, L. C., *J. Am. Chem. Soc.*, **74,** 4216 (1952).
17. Bell, P. H., *J. Am. Chem. Soc.*, **76,** 5565 (1954).

STUDIES ON PITUITARY LACTOGENIC HORMONE

XIV. A SIMPLIFIED PROCEDURE OF ISOLATION*

By R. DAVID COLE† and CHOH HAO LI

(From the Hormone Research Laboratory and the Department of Biochemistry, University of California, Berkeley, California)

(Received for publication, September 27, 1954)

All the methods hitherto published (1–5) for the isolation of hypophyseal lactogenic hormone (prolactin or mammotropin) involve fractional precipitation, which always necessitates submitting the main fraction to the procedure several times in order to obtain a highly purified active preparation. A procedure of this kind is tedious and time-consuming. In the course of further investigations, by counter-current distribution (6), for purity of the hormone protein isolated by the method previously described (4, 5), a simplified and convenient procedure has been developed whereby approximately 2 gm. of prolactin can be obtained from 1 kilo of whole sheep pituitaries.

Procedure for Isolation

Starting Material—A side fraction obtained during the isolation of α-corticotropin (ACTH) from sheep pituitary glands serves as the starting material; this fraction (7, 8) is obtained by dissolving the "acid-acetone powder" (AAP) in water at pH 3 and bringing the solution to 0.06 saturation with respect to NaCl. From 1 kilo of the gland, approximately 20 gm. of this NaCl precipitate (called crude prolactin) may be obtained, having a lactogenic activity of about 5 i.u. per mg. as assayed by the crop sac-stimulating method (9, 10) in month-old Silver King pigeons.

Isoelectric Precipitation—20 gm. of crude prolactin are dissolved in 1 liter of water with the aid of 1.0 M NaOH. The resulting solution (pH 9) is adjusted carefully to pH 6.3 by the addition of 1.0 M HCl and chilled in a cold bath (0–1°) for 1 to 2 hours. The precipitate which forms contains little lactogenic activity and is discarded. The pH of the supernatant fluid is further lowered to 5.6. The precipitate is collected by centrifugation, dissolved in 250 ml. of water by adjusting to pH 8 with 1.0 M NaOH, and lyophilized. The resulting dry white powder (Fraction A) amounts to about 4 gm. and possesses an activity of 20 i.u. per mg.

Counter-Current Distribution—The solvent system employed is 2-butanol-

* Aided in part by a research grant from the Albert and Mary Lasker Foundation.

† Du Pont Postgraduate Fellow in Biochemistry (1953–54).

0.40 per cent dichloroacetic acid (DCA), prepared in the following manner: Distilled water and dry 2-butanol (Shell Chemical Corporation, distilled from $SnCl_2$) in a volume ratio of 1.155:1 are shaken together for equilibration, and the two layers are separated. The lower phase, made up to 0.40 per cent DCA, is shaken with an equal volume of upper phase, and the resulting two-phase system is allowed to equilibrate, with occasional shaking, for at least 24 hours before use.

4 gm. of Fraction A are dissolved in 100 ml. of the lower phase and then mixed with 100 ml. of the organic phase. Counter-current distributions (fifteen transfers) are carried out in a series of centrifuge cups (250 ml.),

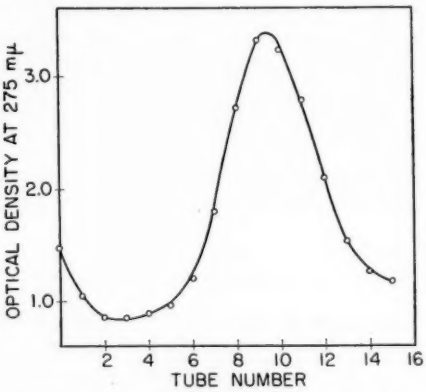


FIG. 1. Counter-current distribution (fifteen transfers) of partially purified prolactin (Fraction A, 4 gm.). Solvent system, 2-butanol-0.4 per cent aqueous dichloroacetic acid.

and occasionally separation of the phases is hastened by centrifugation. A typical distribution pattern may be seen in Fig. 1. The protein concentration in each phase is determined by ultraviolet absorption at 275 mμ in a Beckman spectrophotometer, model DU. The contents of tubes 8 through 11 are combined, dialyzed,[1] and lyophilized. The lyophilized white powder is dissolved in 150 ml. of water at pH 8.0 with the aid of 1.0 M NaOH. Any precipitate which forms is removed by centrifugation; the clear supernatant fluid is then adjusted to pH 5.6 and kept at 0° for 16 to 20 hours. The precipitate is collected by centrifugation and dissolved in 100 ml. of water by adjusting the pH to 7.5. The clear solution is frozen and dried in a vacuum. The lyophilized product (2 gm.), Fraction B, is the lactogenic hormone. The yield and biological potency of

[1] For large scale preparation, it is convenient to remove most of the organic phase before dialysis by evaporation under reduced pressure at room temperature.

the product obtained from each step in the isolation procedure are reported in Table I. It may be noted that practically all of the activity originally present in the acid-acetone extract of sheep pituitary glands is recovered in the final product.

TABLE I

Yield and Potency of Various Fractions Obtained by Simplified Procedure for Isolation of Prolactin

Preparation No.	Procedure	Yield from 1 kilo fresh sheep glands	Estimated potency
		gm.	*i.u. per mg.*
AAP	Acid-acetone extract	30	3
Crude prolactin	NaCl precipitation	20	5
Fraction A	Isoelectric precipitation	4	20
" B	Counter-current distribution	2	35

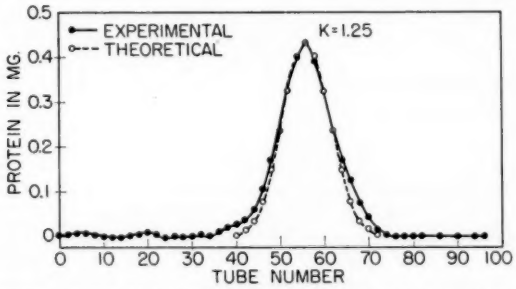


FIG. 2. Counter-current distribution (100 transfers) of lactogenic hormone (Fraction B, 92 mg.). Solvent system, 2-butanol-0.35 per cent aqueous dichloroacetic acid.

Homogeneity Studies

Fig. 2 presents the pattern obtained when Fraction B is submitted to counter-current distribution consisting of 100 transfers in the all-glass apparatus of Craig and Post (11) with the system 2-butanol-0.35 per cent DCA. It may be seen that the distribution pattern appears to follow closely a theoretical curve having a partition coefficient (K) of 1.25. Additional evidence for the homogeneity of Fraction B was provided by studies[2] which included determination of N-terminal amino acid sequence and amino acid composition, as well as sedimentation behavior.

When Fraction B was assayed in month-old pigeons, it was found to contain a crop sac-stimulating potency of 35 i.u. per mg. (Table II). The

[2] A detailed account of these studies will be described elsewhere (see Cole (12)).

luteotropic activity of Fraction B was also determined, according to the procedure of Lyons *et al.* (13), by the extent of stimulation of progestogen secretion produced by the hormone in hypophysectomized rats as evidenced by deciduomal formation. As expected, the hormone protein was found to possess such activity (Table III).

TABLE II

Crop Sac-Stimulating Activity of Lactogenic Hormone (Fraction B)

Preparation	Total dose	No. of pigeons	Crop sac weight	Estimated potency
	mg.		*gm.*	*i.u. per mg.*
Control		6	1.49 ± 0.29*	
International standard†	1.0	6	2.26 ± 0.26	
	2.0	20	2.92 ± 0.16	
	3.0	15	3.91 ± 0.23	
	5.0	15	5.07 ± 0.33	
Fraction B	0.67	24	3.08 ± 0.04	34
	0.82	21	3.73 ± 0.18	36
	1.00	27	4.57 ± 0.18	39

* Mean ± standard error.

† Defined as 10 i.u. per mg.

TABLE III

Luteotropic Activity of Lactogenic Hormone (Fraction B)

Preparation	Total dose	No. of rats*	Positive deciduoma
	mg.		
Control		10	0
Fraction B	1.40	3	3
	0.70	5	5
	0.35	6	4
Progesterone	2.0	5	5
	1.0	3	3

* The animals were immature female rats (Long-Evans strain) hypophysectomized at 30 days of age and given daily injections for 1 week thereafter.

A total dose of 14 mg. of Fraction B administered to immature female rats (30 days of age) over a period of 7 days beginning the day of hypophysectomy produced no histological evidence[3] for the presence of thyrotropic, gonadotropic, or adrenocorticotropic activity. According to the adrenal ascorbic acid depletion assay technique (14, 15) in hypophysecto-

[3] The authors wish to thank Dr. W. R. Lyons and Miss Ruth Johnson for performing the histological studies of these tissues.

mized rats, it was estimated that 200 γ of Fraction B possessed an ACTH contamination equivalent to approximately 0.1 γ of α-corticotropin. A total dose of 10 mg. in 4 days caused no increment of the tibia width of hypophysectomized female rats as determined by the tibia test (16), indicating that the preparation is essentially free from the contamination of growth hormone. In hypophysectomized frogs (*Rana pipiens*), no evidence of intermedin activity was observed at a dose level of 25 γ.

SUMMARY

A simplified procedure has been described for the isolation of lactogenic hormone (prolactin) from an acid-acetone extract of sheep pituitary glands. From 1 kilo of whole pituitaries, approximately 2 gm. of the hormone may be obtained; it possesses a crop sac-stimulating activity of 35 i.u. per mg. and is also potent with respect to luteotropic action. The preparation is essentially free from growth hormone, ACTH, thyrotropin, the gonadotropins, and intermedin. The hormone protein distributes as a single component in 2-butanol-0.35 per cent aqueous dichloroacetic acid through 100 transfers and has a partition coefficient of 1.25. Other physicochemical investigations also indicate that the hormone behaves as a homogeneous protein.

BIBLIOGRAPHY

1. White, A., Catchpole, H. R., and Long, C. N. H., *Science*, **86,** 82 (1937).
2. Lyons, W. R., *Cold Spring Harbor Symposia Quant. Biol.*, **5,** 198 (1937).
3. Bates, R. W., and Riddle, O., in Allen, E., Sex and internal secretion, Baltimore, 2nd edition, 1093 (1939).
4. Li, C. H., Lyons, W. R., and Evans, H. M., *J. Biol. Chem.*, **140,** 43 (1941).
5. Li, C. H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, **146,** 627 (1942).
6. Craig, L. C., and Craig, D., in Weissberger, A., Technique of organic chemistry, New York, **3,** 200 (1950).
7. Li, C. H., *Acta endocrinol.*, **10,** 255 (1952).
8. Li, C. H., Geschwind, I. I., Levy, A. L., Harris, J. I., Dixon, J. S., Pon, N. G., and Porath, J. O., *Nature*, **173,** 251 (1954).
9. Riddle, O., Bates, R. W., and Dykshorn, S. W., *Am. J. Physiol.*, **105,** 191 (1933).
10. Hall, S. R., *Endocrinology*, **34,** 1 (1944).
11. Craig, L. C., and Post, H. O., *Anal. Chem.*, **21,** 500 (1949).
12. Cole, R. D., Dissertation, University of California (1954).
13. Lyons, W. R., Li, C. H., Johnson, R. E., and Cole, R. D., *Proc. Soc. Exp. Biol. and Med.*, **84,** 356 (1953).
14. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, **42,** 379 (1949).
15. Geschwind, I. I., Williams, B. S., and Li, C. H., *Acta endocrinol.*, **8,** 247 (1951).
16. Greenspan, F. S., Li, C. H., Simpson, M. E., and Evans, H. M., *Endocrinology*, **45,** 455 (1949).

XUM

GROWTH AND ENZYMATIC ACTIVITIES OF VITAMIN B_6 ANALOGUES

I. D-ALANINE SYNTHESIS

By JOANNE OLIVARD and ESMOND E. SNELL

(From the Biochemical Institute and the Department of Chemistry, The University of Texas, and the Clayton Foundation for Research, Austin, Texas)

(Received for publication, August 12, 1954)

It is generally accepted that structural analogues of a given metabolite exert their stimulatory or inhibitory effects upon growth by virtue of their ability either to substitute for the metabolite in metabolism or to interfere with its normal utilization. Experiments showing such effects in growing organisms and with isolated enzymes are frequently reported. In few if any cases, however, have the effects of these analogues on growing organisms been correlated with their effects upon the particular enzymatic reaction which is limiting the growth of the organism.

Experiments of this latter nature conducted with *Streptococcus faecalis* and some structural analogues of vitamin B_6 are described in this and the following paper. This organism grows well in the absence of vitamin B_6 and synthesizes little if any of this vitamin in a suitable medium containing each of the protein-derived L-amino acids together with D-alanine (1, 2). D-Alanine and several L-amino acids are non-essential (*i.e.* can be synthesized) when vitamin B_6 is supplied to the organism. Several of the amino acids that appear essential can be replaced in the presence of vitamin B_6 (but not in its absence) by the corresponding keto acids, which are converted to the amino acids by transamination (2). The transaminases involved in these latter conversions and the racemase involved in synthesis of D- from L-alanine are known enzymes and can be obtained conveniently in cell-free form (3, 4). By omitting a suitable amino acid from the medium, growth can thus be made dependent upon its synthesis, which in some instances occurs through action of a single known vitamin B_6-dependent enzyme obtainable in cell-free form. The effects of structural analogues of vitamin B_6 on the growing system and on the cell-free enzyme can then be compared.

In the experiments described below, synthesis of D-alanine by the cells has been made the growth-limiting reaction, and the effects of compounds related to vitamin B_6 on growth and on the activity of the alanine racemase have been compared. In Paper II (5), additional comparisons of this same nature but involving different enzymes are made.

XUM

EXPERIMENTAL

Materials and Methods—Pyridoxal, pyridoxamine, and pyridoxine were commercial products. Pyridoxal phosphate and pyridoxamine phosphate were synthesized as described by Peterson *et al.* (6). Synthesis of the ω-methyl analogues of each of these five compounds, in which the 2-methyl group of vitamin B_6 has been replaced by an ethyl group, is described elsewhere (7). 5-Deoxypyridoxal (8, 9), 5-deoxypyridoxamine, 4-deoxypyridoxine, and 4-deoxypyridoxine phosphate were gifts from Dr. Karl Folkers. The preparation and certain properties of 4-nitrosalicylaldehyde have been described (10). Other materials were from commercial sources.

Growth tests in which the biosynthesis of D-alanine limits growth were carried out with *S. faecalis* R (ATCC 8043) essentially as described by Rabinowitz and Snell (11, 12). The vitamin B_6-free medium of Kihara *et al.* (13) was used, except that the sodium acetate, sodium bicarbonate, ascorbic acid, L-cystine, purine bases, uracil, and DL-alanine were omitted. L-Alanine was added at a level of 750 γ per 3 ml. of double strength medium unless otherwise indicated. After autoclaving and cooling, each 3 ml. of the double strength medium were supplemented aseptically with 0.5 ml. of a previously autoclaved solution of L-cysteine hydrochloride (2 mg. per ml.), inoculated, and 2 ml. of the resulting medium added to each culture tube containing the sterile samples (12) in 2.5 ml. of water. Assays were then incubated at 37° for the desired time.

Cells for use in the preparation of alanine aporacemase were grown in this same medium, modified by substitution of acid-hydrolyzed casein (14) for all of the amino acids except tryptophan and cystine and supplemented with DL alanine (1 mg. per 10 ml.). The cell-free apoenzyme was obtained by sonic disintegration and centrifugation, as described by Wood and Gunsalus (4). The supernatant liquid was brought to saturation with solid ammonium sulfate. The precipitated proteins, which contained the aporacemase, were separated by centrifugation and dissolved in approximately 10 ml. of 0.1 M phosphate buffer, pH 8.1, for each gm. of original cells. The racemization of L-alanine was followed manometrically by measuring the rate of oxygen uptake in the presence of an excess of D-amino acid oxidase (4, 15). The aporacemase was equilibrated with coenzyme and inhibitors for 15 minutes at 37° before addition of L-alanine, and oxygen uptake was followed for 30 minutes beginning 10 or 15 minutes after the addition of substrate. The uptake of 1 μl. of oxygen in this procedure corresponds to the production of 0.089 μmole (8.0 γ) of D-alanine.

Effect of ω-Methyl Analogues of Vitamin B_6 on Growth of S. faecalis—The effects on growth of *S. faecalis* of supplementing the basal medium with pyridoxal, ω-methylpyridoxal, or D-alanine are shown in Table I. The shapes of the dose-response curves to pyridoxal and to ω-methylpyridoxal

are similar, but the latter compound is only 3 to 6 per cent as active on a molar basis as the former. Previous work (16) has established that vitamin B_6 allows growth under these conditions by permitting synthesis of D-alanine; in the absence of vitamin B_6, externally supplied D-alanine is utilized for growth, and the cells thus obtained are extremely low in the vitamin (17). The only reaction in this system, therefore, for which external supplies of vitamin B_6 are required is the synthesis of D-alanine, and analogues of vitamin B_6 that stimulate or inhibit growth in the absence of added D-alanine must do so by affecting its synthesis directly or indirectly.[1] Pyridoxal promotes growth by conversion to pyridoxal phosphate, which activates the alanine racemase (4), permitting synthesis of D-alanine from the L-alanine supplied with the medium. ω-Methylpyridoxal promotes growth in place of pyridoxal, and, since its conversion to pyridoxal is

TABLE I

Comparative Growth Response of S. faecalis to Pyridoxal, ω-Methylpyridoxal, and D-Alanine

	Supplement						
	Pyridoxal			ω-methylpyridoxal		D-Alanine	
mμmoles per 6 ml	0	0.003	0.010	0.10	0.30	4000	6000
Dry cells per ml., *mg.**	0	0.72	1.2	0.76	1.1	0.64	1.2

* Incubation time, 15 hours.

extremely unlikely on chemical grounds,[2] the results indicate that it is phosphorylated by the cells and that the resulting ω-methylpyridoxal phosphate activates the alanine racemase. Either absorption of the analogue, its conversion to the corresponding phosphate, or activation of the racemase by the latter compound must proceed with lowered effectiveness to explain the lowered growth-promoting activity of the analogue compared with that of the vitamin. In Table II the growth-promoting activities of the four forms of vitamin B_6 active for *S. faecalis* are compared with those

[1] Cells grown with D-alanine in the absence of vitamin B_6 contain vanishingly small amounts of the vitamin (17). It is possible, however, that these amounts are essential for growth. Any function served by them, however, should be insensitive to inhibition by vitamin analogues, since such traces of the vitamin, if present, arise by synthesis, and all organisms so far investigated that synthesize their vitamin B_6 are extremely resistant to the inhibitory action of vitamin B_6 analogues (12).

[2] Appropriate differential assays with yeast, for which the ω-methyl analogues of vitamin B_6 are inactive, and with *Lactobacillus casei*, for which ω-methylpyridoxal is highly active, show that cells grown with the analogue contain the analogue and do not convert it to pyridoxal (Sandman, R. P., and Snell, E. E., unpublished data).

XUM

of the corresponding ω-methyl analogues. Each of the analogues supports growth, and the similarity in ratios of amounts of the parent compound to amounts of the corresponding analogue required for growth indicates that phosphorylation of the analogues by the cells is not limiting their activity.

Previous studies have shown that 4-deoxypyridoxine does not inhibit growth of *S. faecalis* in media lacking only D-alanine and supplied with vitamin B_6 (12), whereas 5-deoxypyridoxal and 5-deoxypyridoxamine are inhibitory (9). These results should reflect the behavior of these compounds toward the alanine racemase.

TABLE II

Comparative Activities of Vitamin B_6 and Its ω-Methyl Analogues in Promoting Growth of S. faecalis in Absence of D-*Alanine*

Compound added	Amount required for half maximal growth*	Relative activity	Compound added	Amount required for half maximal growth*	Relative activity
	mμmole per 6 ml.			*mμmole per 6 ml.*	
Pyridoxamine	0.0055	44	ω-Methylpyridoxamine	0.081	3.0
Pyridoxal	0.0024	100	ω-Methylpyridoxal	0.081	3.0
Pyridoxamine phosphate	0.0024	100	ω-Methylpyridoxamine phosphate	0.038	6.3
Pyridoxal phosphate	0.038	6.3	ω-Methylpyridoxal phosphate	0.81	0.3

* Incubation time, 15 to 16 hours.

Activation of Alanine Racemase by ω-Methylpyridoxal Phosphate—From the Lineweaver-Burk (18) plots of Fig. 1, it is seen that ω-methylpyridoxal phosphate (Fig. 1, *B*) activates the alanine racemase, as predicted from the growth experiments. The Michaelis constant (K_{Co}) for pyridoxal phosphate calculated from Fig. 1, *A* is 4.5×10^{-7}; the average[3] of five determinations was 4.4×10^{-7}. The corresponding figure for ω-methylpyridoxal phosphate calculated from Fig. 1, *B* is 3.5×10^{-6}; the average of five determinations was 4.5×10^{-6}. On the molar basis, therefore, the ω-methyl analogue is approximately one-tenth as active as pyridoxal phosphate in activation of the enzyme.

[3] Analytically pure pyridoxal phosphate was used in these determinations. Wood and Gunsalus (4) reported a K_{Co} value (2×10^{-6}) about 4 times as high as our figure. Their work was done before pure pyridoxal phosphate became available. A further difference of undetermined significance lies in their use of a 5 minute preincubation period between apoenzyme and coenzyme before measurement as opposed to our use of a 15 minute period. Full association between pyridoxal phosphate and certain apoenzymes requires longer times than had been realized previously (20).

Extrapolation of the curves of Fig. 1 through the y axis shows that the maximal velocity (V_M) achieved with the holoenzyme is less for ω-methylpyridoxal phosphate activation than for pyridoxal phosphate activation. To clarify this relationship, and to check the possibility that the value for K_{Co} might vary with the concentration of L-alanine used, the effect of substrate concentration on the values for K_{Co} and V_M was determined (Table III). Each horizontal line of Table III represents simultaneous determinations under like conditions; however, for each level of L-alanine the amount of racemase preparation was not necessarily the same, and

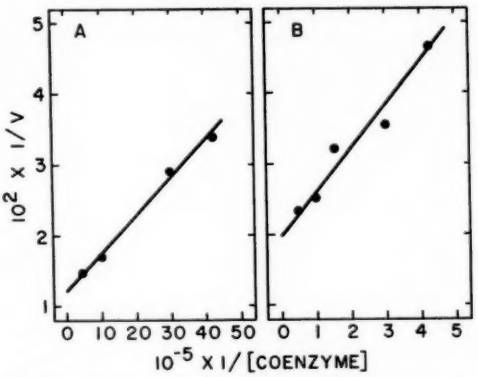


FIG. 1. The relation of coenzyme concentration to velocity of racemization of L-alanine by alanine aporacemase. V = microliters of oxygen absorbed in 30 minutes. [Coenzyme] = concentration in moles per liter. *A*, pyridoxal phosphate; *B*, ω-methylpyridoxal phosphate. Each Warburg vessel contained 0.2 ml. of 20 per cent KOH in the well, 0.3 ml. of the D-amino acid oxidase preparation and 0.3 ml. of 0.5 M L-alanine in the side arm, and 0.3 ml. of 0.5 M phosphate buffer (pH 8.3), 0.3 ml. of the alanine aporacemase preparation, coenzyme, and water in the main compartment to make a total volume of 3.2 ml. For other details, see the text.

hence absolute values of $1/V_M$ have no significance. The results show that the K_{Co} values do not vary with L-alanine concentration. However, the maximal rate for the ω-methylpyridoxal phosphate-activated racemase approaches the value obtained with pyridoxal phosphate activation (*i.e.* $V_M'/V_M \rightarrow 1.0$) as the concentration of L-alanine is increased. Testing of higher concentrations of L-alanine was not experimentally feasible.

This behavior must reflect a lower affinity of the holoenzyme for the substrate when the modified coenzyme (ω-methylpyridoxal phosphate) is the activator. Values for the apparent Michaelis constant (K_S) for the L-alanine-holoracemase dissociation were calculated from the plots of Fig. 2. With pyridoxal phosphate as coenzyme, K_S is 9.8×10^{-3}; with ω-methylpyridoxal phosphate as coenzyme, the corresponding value is 19.0×10^{-3}.

These findings indicate that the low activity of ω-methylpyridoxal (as compared to pyridoxal) in promoting growth of *S. faecalis* (Table II) might be the result of two factors: (*a*) the lower affinity of its phosphate for the aporacemase, and (*b*) the lower affinity of the resulting holoracemase for

TABLE III

Effect of Concentration of L-*Alanine upon Coenzyme-Aporacemase Dissociation and upon Maximal Rate of Racemization*

L-Alanine concentration	Aporacemase + pyridoxal phosphate		Aporacemase + ω-methylpyridoxal phosphate		$\frac{V_M'}{V_M}$
M × 10^2	K_{Co} × 10^7	$1/V_M$	K_{Co}' × 10^7	$1/V_M'$	
4.0	4.2	0.0135	44	0.0290	0.47
5.0	4.5	0.0124	35	0.0200	0.62
6.7	4.3	0.0127	39	0.0188	0.68

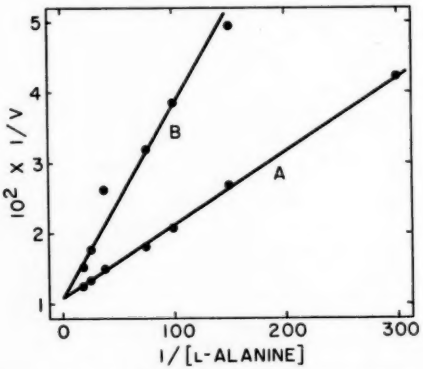


FIG. 2. The relation of reaction velocity (V) to concentration of L-alanine for holoracemase activated by pyridoxal phosphate or by ω-methylpyridoxal phosphate. Curve A, pyridoxal phosphate, 2.0×10^{-6} M; Curve B, ω-methylpyridoxal phosphate, 6.7×10^{-5} M. Other conditions were similar to those in Fig. 1, but the concentration of L-alanine was varied. V = microliters of oxygen absorbed in 30 minutes.

the substrate, L-alanine. The activities of pyridoxal and ω-methylpyridoxal were therefore compared in a medium containing varying amounts of L-alanine (Table IV). In accord with expectation from the low K_S values of the holoenzymes, the absolute activities of both pyridoxal and ω-methylpyridoxal increased with increasing amounts of L-alanine; that of the ω-methyl analogue, however, was increased most. With sufficient L-alanine present, its activity approached 10 per cent that of pyridoxal, which would

appear to be a limiting value set by the comparative affinities of the two coenzymes for the aporacemase. The result demonstrates that the growth-promoting activity of the ω-methyl analogues in the medium used here is limited only by the ability of ω-methylpyridoxal phosphate to activate the

TABLE IV

Effect of Concentration of L-*Alanine on Growth-Promoting Activities of Pyridoxal and ω-Methylpyridoxal for S. faecalis*

L-Alanine	Amount required for half maximal growth*	
	Pyridoxal	ω-Methylpyridoxal
γ per 6 ml.	*mμmole per 6 ml.*	*mμmole per 6 ml.*
500	0.015	0.34
750	0.007	0.10
1000	0.006	0.08
1250	0.005	0.06
1500		0.06

* Incubation time, 13 hours.

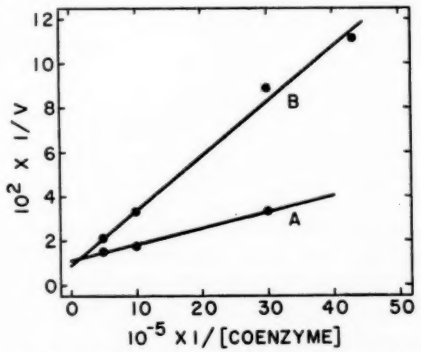


FIG. 3. Inhibition of alanine racemization by 5-deoxypyridoxal. Curve A, no inhibitor; Curve B, 5-deoxypyridoxal (2.33×10^{-4} M). Assay conditions as in Fig. 1. Coenzyme = pyridoxal phosphate.

alanine aporacemase, and not by the absorption of the analogues by the cell or by their conversion to ω-methylpyridoxal phosphate.

Effects of Other Vitamin B_6 Analogues on Alanine Racemase—As shown in Fig. 3, 5-deoxypyridoxal inhibits competitively the activation of alanine aporacemase by pyridoxal phosphate. The enzyme-inhibitor dissociation constant (K_I) calculated from Fig. 3 is 8.9×10^{-5}. Similar data were obtained with several other analogues of vitamin B_6, all of which proved to inhibit competitively as judged from the Lineweaver-Burk plots. These

XUM

plots were used to calculate the K_I values summarized in Table V. Of the compounds tested, 4-nitrosalicylaldehyde was the most potent inhibitor, followed closely by 5-deoxypyridoxal. As pointed out elsewhere (19), these compounds are very similar to pyridoxal and to pyridoxal phosphate in electronic configuration and reactivity toward amino acids. In contrast to pyridoxal phosphate, pyridoxal and other unphosphorylated forms of the vitamin inhibit enzymatic activity. Thus the phosphate ester group, although essential for formation of a catalytically active complex, is not essential for combination with the apoenzyme. Its contribution to such combination, however, is emphasized by the fact that all of the inhibitors exhibit a much lower affinity for the aporacemase than does either pyridoxal phosphate or ω-methylpyridoxal phosphate.

TABLE V

*Enzyme-Inhibitor Dissociation Constants (K_I) for Alanine Racemase of S. faecalis**

Inhibitor	K_I
4-Nitrosalicylaldehyde	4.0×10^{-5}
5-Deoxypyridoxal	8.9×10^{-5}
Pyridoxal	3.2×10^{-4}
ω-Methylpyridoxal	5.3×10^{-4}
Pyridoxamine	5.9×10^{-4}
ω-Methylpyridoxamine	2.7×10^{-3}
Pyridoxine	2.3×10^{-2}

* Conditions of determination as in Fig. 1.

Several of the inhibitors, *e.g.* 4-deoxypyridoxine phosphate and, to a lesser extent, 4-deoxypyridoxine, are inhibitory only if incubated with the apoenzyme before addition of pyridoxal phosphate (Table VI). Their behavior in this respect contrasts with that of 5-deoxypyridoxal, which is inhibitory with or without preincubation, although to a different extent, depending upon the order of addition of coenzyme and inhibitor. Association of the inhibitors with the apoenzyme, like that of the coenzyme (20), is obviously not instantaneous and not freely reversible. Under conditions of simultaneous addition, inhibition by 4-deoxypyridoxine or its phosphate would be expected only at extremely high levels, since the K_I values of these compounds must be higher than any in Table V. Similar behavior of 4-deoxypyridoxine phosphate and pyridoxine phosphate toward the glutamic-aspartic transaminase of hog heart has been noted (20).

Correlation of Effects of Inhibitory Analogues on Alanine Racemase and upon Growth—The effects of several analogues of vitamin B_6 on the growth of *S. faecalis* were examined by Rabinowitz and Snell (9, 12) under condi-

tions such that D-alanine was the growth-limiting reaction. Neither 4-deoxypyridoxine[4] nor ω-methylpyridoxine inhibited growth. Since in such experiments the inhibitor and vitamin are presented simultaneously to the growing organism, the result is that expected from the observed effect of 4-deoxypyridoxine (or 4-deoxypyridoxine phosphate) on the cell-free racemase (Table VI).

In contrast to these analogues, 5-deoxypyridoxal competes with the coenzyme for alanine racemase when the two are added simultaneously

TABLE VI

Effect of Order of Addition of Coenzyme and Inhibitors on Activity of Alanine Racemase

Inhibitor	μmoles per vessel	Order of addition to apoenzyme*	Per cent inhibition
5-Deoxypyridoxal	3	Preincubation with inhibitor	81.3
		" " coenzyme	29.2
		Simultaneous addition	46.3
4-Deoxypyridoxine	4	Preincubation with inhibitor	42.8
		" " coenzyme	2.5
		Simultaneous addition	2.2
4-Deoxypyridoxine phosphate	0.8	Preincubation with inhibitor	57.8
		" " coenzyme	−2.0
		Simultaneous addition	−1.0

* The assay was carried out in two arm Warburg vessels containing 0.2 ml. of 20 per cent KOH in the well, 0.2 ml. of 0.4 M L-alanine and 0.5 ml. of D-amino acid oxidase preparation in one side arm, 0.4 ml. of 1.0×10^{-3} M pyridoxal phosphate (0.4 μmole per vessel) and inhibitor in the main compartment or side arm (as indicated by the order of addition), and 5 mg. of acetone-dried cells, 0.3 ml. of 0.5 M phosphate buffer, pH 8.3, and water to make a total volume of 3.2 ml. in the main compartment. Inhibitor or coenzyme equilibrated with the racemase for 10 minutes; the second addition also equilibrated for 10 minutes, the contents of the second side arm containing substrate and oxidase added, and the oxygen uptake followed after 10 minutes as noted in the text.

(Table VI); correspondingly, this analogue inhibits growth of *S. faecalis* under conditions in which D-alanine synthesis limits growth (9), and this inhibition is competitive with vitamin B_6. Although the effects of this compound on the alanine racemase thus suffice to explain its effect on growth, they do not exclude its action at other loci as well, *e.g.* in the competitive inhibition of pyridoxal phosphate synthesis.

4-Nitrosalicylaldehyde behaves like 5-deoxypyridoxal toward alanine

[4] The effects of 4-deoxypyridoxine and of 4-deoxypyridoxine phosphate on growth in the medium used here were checked. In confirmation of previous results, neither was inhibitory at ratios (inhibitor to pyridoxamine) of 2.4×10^5 and 4.8×10^4, respectively.

XUM

racemase and, as expected, inhibits growth of *S. faecalis* at rather low levels. However, this inhibition is not competitive with vitamin B_6 and, in contrast to that by 5-deoxypyridoxal (9), is not alleviated by addition of D-alanine (Table VII). Apparently some function unrelated to vitamin B_6 is inhibited irreversibly at concentrations below those which suffice to stop growth by interfering with D-alanine synthesis.

Although the various unphosphorylated forms of vitamin B_6 and their ω-methyl analogues inhibit the cell-free racemase, they promote growth of the living organism, and this is adequate proof of their conversion to pyridoxal phosphate or its ω-methyl analogue. Since they are converted

TABLE VII

*Inhibition of S. faecalis by 4-Nitrosalicylaldehyde**

4-Nitrosalicylaldehyde, μmoles per 6 ml.	Supplement			
	Pyridoxamine†		Pyridoxamine† phosphate	D-alanine‡
	mμmoles per 6 ml.			
	0.083	0.167	0.070	4.2×10^3
	Mg. dry cells per ml.			
0.000	1.50	1.43	1.50	1.32
0.375	1.18	1.21	1.33	1.33
0.500	0.91	0.84	1.05	0.84
0.700	0.30			0.30
1.00	0.07	0.07	0.26	0.12
1.30	0.04	0.07	0.06	0.07

* Incubation time, 13 hours.

† With 750 γ of L-alanine per 6 ml.

‡ With 375 γ of L-alanine (750 γ of DL-alanine) per 6 ml.

in the cell to the very products that counteract their inhibitory effects, failure to obtain inhibition of growth with them is the result to be expected.

DISCUSSION

These results show that, when the enzymatic reaction limiting growth of an organism is known, the effects of specific antimetabolites on growth are closely predictable from their effects upon that enzymatic reaction *in vitro*. Instances in which such reactions are known and which permit correlations of the type demonstrated here are quite rare; because of this, the present experimental system may be useful in a variety of other studies. Since different enzymes differ in their properties, it should be expected that

the relationships to growth of *S. faecalis* of the compounds studied here might be changed materially by changing the conditions of growth in a way that would make activity of another enzyme the growth-limiting reaction. That this is true is demonstrated in Paper II; the effects of ω-methylpyridoxal on *S. faecalis* range from growth promotion to growth inhibition, depending upon the exact conditions (5).

The pronounced decrease in the affinity of the alanine racemase for its substrate when pyridoxal phosphate is replaced by ω-methylpyridoxal phosphate is an effect of a type not previously observed. It demonstrates that the coenzyme, as well as the protein, is of importance for formation of the enzyme-substrate complex. The result is easily rationalized; any deformation in structure of the enzyme at its active centers, such as would be produced by combination with a coenzyme of modified structure, would be expected to change the ability to bind the substrate. This is true even though, as in the present instance, the modified coenzyme does not differ materially in chemical properties from the naturally occurring compound.

4-Deoxypyridoxine has been widely used as a vitamin B_6 antagonist in higher animals. If the affinities of the enzymes of higher animals resemble those of the alanine racemase for vitamin B_6 analogues, 5-deoxypyridoxal should be a much more potent antagonist for such studies, provided it is not further metabolized by the intact animal.

SUMMARY

Growth conditions were arranged to make synthesis of D- from L-alanine by the alanine racemase the growth-limiting reaction in *Streptococcus faecalis*. The effects of a variety of compounds related to vitamin B_6 upon growth and upon the activity of the cell-free alanine racemase were then determined.

Under these conditions, ω-methylpyridoxal, ω-methylpyridoxamine, and their 5-phosphates promoted excellent growth of this organism; on the molar basis they were 3 to 8 per cent as active as the corresponding forms of vitamin B_6 (pyridoxal, etc.). Similarly, ω-methylpyridoxal phosphate fully activates the alanine racemase of this organism, but its affinity for the aporacemase is only approximately one-tenth that of pyridoxal phosphate.

The maximal rate of racemization obtained with ω-methylpyridoxal phosphate as coenzyme is lower than that obtained with pyridoxal phosphate, but approaches the latter as the concentration of L-alanine is increased. Suitable measurements showed that the affinity of L-alanine for the ω-methylpyridoxal phosphate-apoenzyme complex was about half as great as for the pyridoxal phosphate-aporacemase complex. Corresponding growth experiments showed that the activity of ω-methylpyridoxal relative to that of pyridoxal was increased by increasing the L-alanine con-

XUM

tent of the medium and approached the theoretical limit set by the comparative affinities of coenzyme and analogue-coenzyme for the aporacemase.

Activation of the cell-free racemase by pyridoxal phosphate is inhibited competitively by 4-nitrosalicylaldehyde, 5-deoxypyridoxal, pyridoxal, pyridoxamine, ω-methylpyridoxal, ω-methylpyridoxamine, and, much less strongly, by pyridoxine. This effect of 5-deoxypyridoxal on the racemase is a sufficient, although not necessarily a complete, explanation for its inhibitory effects on growth of *S. faecalis*, which are prevented competitively by vitamin B_6 and non-competitively by D-alanine (9). 4-Nitrosalicylaldehyde also inhibits growth, but its effects are not counteracted by vitamin B_6 or D-alanine; it inhibits, therefore, by a mechanism other than (or in addition to) its effects on the racemase. Inhibition of growth by the various forms of vitamin B_6 and its ω-methyl analogues was not expected, since these compounds are converted in the cell to coenzymes that counteract their inhibitory effect.

4-Deoxypyridoxine and 4-deoxypyridoxine phosphate do not inhibit growth under the conditions described here. Similarly, they do not inhibit activation of alanine aporacemase by pyridoxal phosphate when analogue and coenzyme are added simultaneously.

Certain aspects and implications of these findings are discussed.

BIBLIOGRAPHY

1. Snell, E. E., and Guirard, B. M., *Proc. Nat. Acad. Sc.*, **29,** 66 (1943).
2. Holden, J. T., Wildman, R. B., and Snell, E. E., *J. Biol. Chem.*, **191,** 559 (1951).
3. Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, **161,** 311 (1945).
4. Wood, W. A., and Gunsalus, I. C., *J. Biol. Chem.*, **190,** 403 (1951).
5. Olivard, J., and Snell, E. E., *J. Biol. Chem.*, **213,** 215 (1955).
6. Peterson, E. A., Sober, H. A., and Meister, A., *Biochem. Preparations*, **29,** 34 (1953).
7. Ikawa, M., and Snell, E. E., *J. Am. Chem. Soc.*, **76,** 637 (1954).
8. Heyl, D., Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, **75,** 653 (1953).
9. Rabinowitz, J. C., and Snell, E. E., *Arch. Biochem. and Biophys.*, **43,** 408 (1953).
10. Ikawa, M., and Snell, E. E., *J. Am. Chem. Soc.*, **76,** 653 (1954).
11. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **169,** 631 (1947).
12. Rabinowitz, J. C., and Snell, E. E., *Arch. Biochem. and Biophys.*, **43,** 399 (1953).
13. Kihara, H., Klatt, O. A., and Snell, E. E., *J. Biol. Chem.*, **197,** 801 (1952).
14. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139,** 675 (1941).
15. Olivard, J., Metzler, D. E., and Snell, E. E., *J. Biol. Chem.*, **199,** 669 (1952).
16. Holden, J. T., and Snell, E. E., *J. Biol. Chem.*, **178,** 799 (1949).
17. Holden, J. T., Furman, C., and Snell, E. E., *J. Biol. Chem.*, **178,** 789 (1949).
18. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
19. Metzler, D. E., Ikawa, M., and Snell, E. E., *J. Am. Chem. Soc.*, **76,** 648 (1954).
20. Meister, A., Sober, H. A., and Peterson, E. A., *J. Biol. Chem.*, **206,** 89 (1954).

GROWTH AND ENZYMATIC ACTIVITIES OF VITAMIN B_6 ANALOGUES

II. SYNTHESIS OF MISCELLANEOUS AMINO ACIDS

By JOANNE OLIVARD and ESMOND E. SNELL

(From the Biochemical Institute and the Department of Chemistry, The University of Texas, and the Clayton Foundation for Research, Austin, Texas)

(Received for publication, August 12, 1954)

In Paper I (1) conditions were defined in which synthesis of D-alanine by the vitamin B_6-dependent alanine racemase was the growth-limiting reaction in *Streptococcus faecalis*. Under these conditions, a close correlation was observed between the effect of analogues of vitamin B_6 upon growth and their effects on the cell-free racemase.

By adding an excess of D-alanine to this medium, thus making its synthesis unnecessary, and omitting another amino acid, synthesis of this latter amino acid becomes the growth-limiting reaction. Such synthesis requires addition of vitamin B_6 and, in some but not all cases, of the α-keto acid corresponding in structure to the omitted amino acid (2). In the latter event, the keto acids are transformed to the amino acids by transamination (2).

In this study, leucine, phenylalanine, or tyrosine has been omitted from the growth medium, thus making synthesis of this amino acid from the corresponding α-keto acid growth limiting. The effect of certain analogues of vitamin B_6 on growth under these conditions has been compared with their effects on the corresponding cell-free transaminases. Finally, the effects of the analogues on growth were determined when aspartic acid, valine, isoleucine, methionine, serine, cysteine, or alanine has been omitted from the medium and when, consequently, synthesis of one of these amino acids by unidentified enzymes limits growth. It is shown that the effects of ω-methyl analogues of vitamin B_6 in this single organism may range from a rather efficient substitution for vitamin B_6 in promoting growth to an active inhibition of growth, depending upon the enzymatic process limiting growth.

Materials and Methods

Growth Tests—The medium and assay procedure used with *S. faecalis* have been described (1). Except where otherwise noted, 1 mg. of DL-alanine was added per 6 ml., and the amino acid under study was omitted from the medium. When these were required for growth, neutralized solutions of the appropriate keto acids were sterilized by filtration and add-

ed aseptically to test cultures. Departures from these procedures are noted in Tables I to VI.

Keto Acids—The sources of most chemicals have been specified (1). DL-α-Hydroxy-β-methylthiobutyric acid was obtained from the Monsanto Chemical Company. The synthesis of α-hydroxy-β-methylvaleric acid and α-ketoisovaleric acid has been described earlier (2, 3). Sodium pyruvate[1] was prepared by the method of Price and Levintow (4), sodium α-ketoisocaproate[1] by the enzymatic oxidation of leucine (5), α-ketoglutaric acid by transamination between glutamate and glyoxylate (6), oxalacetic acid by hydrolysis of sodium diethyl oxalacetate (7), and phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid by the hydrolysis of the corresponding azlactones (8).

Enzyme Preparations—Cells of *S. faecalis* were grown at 37° in the complete basal medium without vitamin B_6. Between 8 and 12 hours of incubation, the medium was neutralized aseptically with 4 N NaOH, as recommended by Lichstein *et al.* (9) for production of glutamic-aspartic transaminase. After about 12 to 13 hours of incubation, the cells were harvested by centrifugation, washed, suspended in approximately 10 ml. of 0.05 M phosphate buffer, pH 8.3, per gm. of dry cells, disrupted by sonic disintegration as described (1, 10) for the alanine racemase, and the cell residues centrifuged. The resulting supernatant solution contained apoenzymes of each of the enzymes studied here.

Enzyme Determinations. (*a*) *Leucine-Glutamic Acid Transaminase*—The reaction

$$\text{Leucine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{glutamate} + \alpha\text{-ketoisocaproate}$$

was followed in the forward direction. The reaction mixture contained 0.3 ml. of 0.5 M phosphate buffer, pH 8.3, 1.0 ml. of the transaminase preparation, water, and coenzyme (pyridoxal phosphate or ω-methylpyridoxal phosphate) to a total volume of 1.8 ml. These were equilibrated for 15 minutes at 37° in a 15 ml. conical centrifuge tube; 0.4 ml. of 0.1 M α-ketoglutarate, pH 8.3, and 0.8 ml. of 0.05 M DL-leucine, pH 8.3, were then added and the contents mixed. The mixture was divided into equal parts (0.6 ml.) in five centrifuge tubes. At intervals of 5 minutes, the reaction in one of the tubes was stopped with 0.2 ml. of 10 per cent perchloric acid, the denatured protein was separated by centrifugation, and a 0.4 ml. aliquot of the supernatant liquid was allowed to react with 1 ml. of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N HCl. After ½ hour, the sample was extracted with 1.5 ml. of ethyl acetate, and about 0.7 ml. of this extract was applied to five or six adjacent areas on Whatman No. 1 filter paper. Each area had previously been treated with 0.002 ml. of 0.2 M phosphate

[1] Prepared by Dr. D. E. Metzler.

buffer, pH 7.0, to neutralize traces of acid in the ethyl acetate extract. The chromatograph was developed overnight with the upper layer of a mixture of butanol-ethanol-water (50:10:40). The keto acid dinitrophenylhydrazones were eluted quantitatively with 0.02 M phosphate buffer, pH 7.0, and an aliquot was transferred to an Evelyn colorimeter tube for the quantitative, colorimetric determination of the keto acid dinitrophenylhydrazone by a procedure similar to that of Metzler and Snell (11, 12). Each aliquot was diluted to 5 ml. with water and the color developed directly by addition of 5 ml. of 2.5 N NaOH. After 10 minutes the transmittance (G) was read on an Evelyn colorimeter with the No. 515 filter. Calibration curves determined with various keto acid dinitrophenylhydrazones gave the following K values, where $K(2 - \log G)$ = micromoles of keto acid per tube: α-ketoglutaric acid 0.635, phenylpyruvic acid 0.660, *p*-hydroxyphenylpyruvic acid 0.633, pyruvic acid 0.458, and α-ketoisocaproic acid 0.500. The amount of each keto acid on the paper and the sum of the two in each sample were calculated. The results were expressed as the fraction of α-ketoisocaproic acid (*i.e.*, α-ketoisocaproate/total keto acid) formed. This was convenient since it made quantitative application of an exact amount of each sample to the paper unnecessary. Since, during transamination, one keto acid is consumed and another keto acid is produced in equimolar amounts, an accurate picture of the total amount of reaction is afforded by this procedure, provided side reactions are negligible.

In control experiments no α-ketoisocaproic acid was formed from leucine in the absence of either α-ketoglutaric acid or pyridoxal phosphate and neither α-ketoglutaric acid nor α-ketoisocaproic acid was degraded by the enzyme preparation. A fraction of 0.006 of α-ketoglutaric acid was formed per 30 minutes when glutamic acid was substituted for α-ketoglutaric acid and leucine was omitted from the above procedure; for analysis, in this case, α-ketoisocaproic acid equimolar to the leucine ordinarily used was added after precipitation of the protein with perchloric acid. Under the usual assay conditions, the glutamate concentration is small and α-ketoglutaric acid is in excess; therefore 0.006 represents the maximal possible error. Errors of this magnitude for the fraction of α-ketoglutaric acid would not appreciably affect the calculations and were neglected.

(*b*) *Phenylalanine-Glutamic Acid Transaminase*—The reaction

$$\text{Phenylalanine} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{glutamate} + \text{phenylpyruvate}$$

was followed from left to right. The procedure was the same as that described for the leucine-glutamic acid transaminase except that 0.4 ml. of 0.1 M L-glutamate, pH 8.3, was substituted for α-ketoglutarate and 0.8 ml. of 0.05 M phenyl pyruvate, pH 8.3, for DL-leucine. The results were ex-

pressed as the fraction of α-ketoglutarate (*i.e.*, α-ketoglutarate/total keto acid) formed per 30 minutes. The values were corrected for the small fraction of α-ketoglutarate formed in the absence of added coenzyme.

(*c*) *Tyrosine-Glutamic Acid Transaminase*—By substituting 0.05 M *p*-hydroxyphenylpyruvate, pH 8.3, for the phenylpyruvate in procedure (*b*), the transamination of *p*-hydroxyphenylpyruvate with glutamate to yield tyrosine and α-ketoglutarate was followed.

(*d*) *Alanine-Glutamic Acid Transaminase*—The procedure was the same as that for the phenylalanine-glutamic transaminase (*b*) with pyruvate in place of phenylpyruvate.

(*e*) *Cysteine Desulfhydrase*—The desulfhydrase activity of the cell-free extract used here as a source of other enzymes was low and was concentrated partially before investigation. For this purpose, the cell-free extract prepared as described earlier was adjusted to pH 8.0, 0.2 volume of 95 per cent ethanol added, and the solution placed in the refrigerator overnight. Acetic acid (1 N) was added to pH 5.3, and, after standing ½ hour the precipitated protein, which contained the enzyme, was collected by centrifugation and dissolved in one-fourth the original volume of 0.05 M phosphate buffer, pH 8.3.

Enzyme activity was determined by mixing 0.4 ml. of this preparation with 0.4 ml. of 0.5 M phosphate buffer, pH 8.3, and 0.2 ml. of water or coenzyme solutions and incubating at 37° for 15 minutes. L-Cysteine (0.2 ml. of 0.01 M) or water (0.2 ml.) was then added and the reaction stopped after 30 minutes by adding 0.2 ml. of 15 per cent perchloric acid. Pyruvate production was determined as the difference between the "total color" developed with dinitrophenylhydrazine, as described by Metzler and Snell (11), due to coenzyme plus pyruvic acid, and the total color developed after extraction of the pyruvic acid dinitrophenylhydrazone with toluene, due to coenzyme alone.

Results

Growth Promotion by ω-Methyl Analogues of Vitamin B_6—The comparative activities of pyridoxamine phosphate and ω-methylpyridoxamine phosphate in promoting growth when leucine is replaced in the medium with α-ketoisocaproic acid (*i.e.*, when leucine synthesis from the keto acid limits growth) are presented in Fig. 1. Similar curves relating growth response to the concentration of pyridoxal, pyridoxamine, and their ω-methyl analogues[2] in the absence of several of the individual amino acids studied were

[2] Pyridoxine and ω-methylpyridoxine promote growth of this and other lactic acid bacteria only by virtue of the fact that incubation of them with the medium yields minute amounts of the corresponding aldehyde and amine. Similarly, pyridoxal phosphate (and ω-methylpyridoxal phosphate) shows only a fraction of the activity of pyridoxamine phosphate, owing presumably to its limited absorption by the cells. The values for the activity of these compounds, therefore, are not tabulated.

obtained and, from these, the concentrations that permit half maximal growth (see Table I). Depending upon the amino acid omitted, the vita-

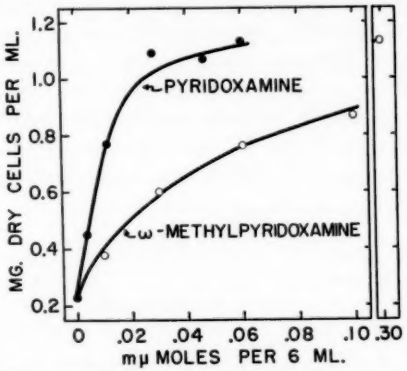


FIG. 1. Comparative activities of pyridoxamine phosphate and ω-methylpyridoxamine phosphate in promoting growth with α-ketoisocaproate replacing leucine. 16 hours incubation.

TABLE I

Comparative Activities of Vitamin B_6 and Its ω-Methyl Analogues in Promoting Growth of S. faecalis under Conditions Requiring Synthesis of Leucine, Phenylalanine, Tyrosine, or Serine

Vitamin B_6 derivative added to medium	Amino acid omitted from medium*								
	None	Leucine		Phenylalanine		Tyrosine		Serine	
	(*a*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Pyridoxamine phosphate	0	0.009	100	0.008	100	0.020	100	0.023	100
"	0	0.030	30	0.010	80	0.015	133	0.032	72
Pyridoxal	0	0.030	30	0.019	42	0.019	105	0.032	72
ω-Methylpyridoxamine phosphate	0	0.041	22	0.085	9.4	0.145	14	0.230	10
ω-Methylpyridoxamine	0	0.118	7.6	0.202	4.0	0.279	7.2	0.420	5.5
ω-Methylpyridoxal	0	0.118	7.6	0.206	3.9	0.482	4.2	0.540	4.3

(*a*), millimicromoles of vitamin B_6 derivative per 6 ml. required for half maximal growth; (*b*), comparative activity, pyridoxamine phosphate = 100.

* The corresponding keto acid is required for growth in the absence of leucine, phenylalanine, or tyrosine (2) and was added at the following levels per 6 ml.: α-ketoisocaproic acid, 0.5 mg.; phenylpyruvic acid, 1.0 mg.; or *p*-hydroxyphenylpyruvic acid, 3.0 mg. Incubation time, 16 hours in all cases.

min B_6 requirement of the organism varies between 3 and 10 times that required when D-alanine synthesis is the growth-limiting reaction ((1); *cf.* (2)). The ω-methyl analogues are from approximately 5 per cent (in D-

alanine synthesis (1)) to as high as 25 per cent (L-leucine synthesis, Table I) as active as the corresponding forms of vitamin B_6, depending upon the reaction in which they participate. Considerable variation in the comparative activity of pyridoxal, pyridoxamine, and pyridoxamine phosphate in the several slightly different media is evident. This must indicate differences in absorption of these compounds or other differences in the efficiency of their utilization by the cells from one medium to another.

Less detailed studies (Table II) show that ω-methylpyridoxamine also supports growth in place of pyridoxamine under conditions requiring synthesis of aspartic acid, methionine, valine, or isoleucine; quantitative determinations of the relative activities were not made.

TABLE II

Activities of Pyridoxamine and ω-Methylpyridoxamine in Promoting Growth of S. faecalis under Conditions Requiring Synthesis of Aspartic Acid, Methionine, Valine, or Isoleucine

The values are in mg. of dry cells obtained per ml. Incubation, 14 hours.

Amino acid omitted	Vitamin B_6 derivative added per 6 ml.		
	None	Pyridoxamine, 0.166 mμmole	ω-Methylpyridoxamine, 2.0 mμmoles
None	1.7		
Aspartic acid* (and asparagine)	0.1	1.23	1.21
Isoleucine†	0.16	0.71	0.60
Methionine‡	0.15	0.52	0.49
Valine§	0.41	1.8	1.8

* 2 mg. of oxalacetic acid added per 6 ml.
† 1 mg. of DL-α-hydroxy-β-methylvaleric acid added per 6 ml.
‡ 2 mg. of DL-α-hydroxy-γ-methylthiobutyric acid added per 6 ml.
§ 2 mg. of α-ketoisovaleric acid added per 6 ml.

Growth Inhibition by ω-Methyl Analogues of Vitamin B_6—If cysteine (and cystine) is omitted from the basal medium used here, its synthesis becomes limiting, and pyridoxamine (or other form of vitamin B_6) is required for growth. ω-Methylpyridoxamine, in contrast to its behavior in each of the systems so far described, failed to promote growth in place of pyridoxamine. Addition of sufficient amounts of ω-methylpyridoxamine to the cysteine-free medium containing pyridoxamine effectively inhibits growth (Fig. 2), and this inhibition was competitively alleviated by pyridoxamine. Since such inhibition does not occur in the cysteine-supplemented medium, some stage of cysteine synthesis apparently is the process inhibited. Unfortunately, details of cysteine synthesis in this organism are not known.

It has been shown that ω-methylpyridoxal phosphate activates the alanine racemase, which allows interconversion of D- and L-alanine, and, correspondingly, permits growth under conditions in which this interconversion is the limiting reaction (1). However, if both D- and L-alanine are omitted from the medium, synthesis of the amino acid by some other route is required. Addition of 100 to 200 γ of DL-alanine per 6 ml. of medium supplied sufficient D-alanine for growth, but not sufficient L-alanine. Under these conditions (Fig. 3) either pyridoxamine or L-alanine, but not D-alanine, permits maximal growth. Also under these conditions, the ω-methyl analogues of vitamin B_6 failed to permit growth in place of pyri-

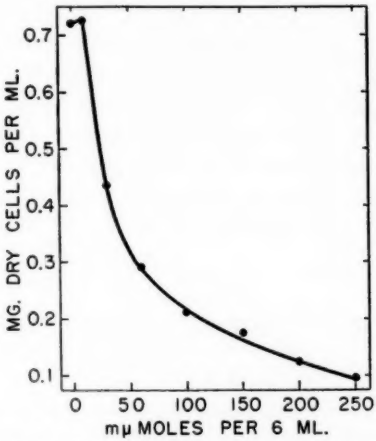


FIG. 2. Inhibition of growth by ω-methylpyridoxamine in a cysteine-free medium. Pyridoxamine, 0.166 mμmole per 6 ml.; 16 hours incubation.

doxamine and inhibited growth when added to the vitamin B_6-containing medium. The inhibition was competitive over the range of concentrations of vitamin B_6 tested (4-fold); inhibition indices for various combinations of inhibitor and vitamin are given in Table III. It is interesting to note that ω-methylpyridoxal is most effective as an inhibitor when pyridoxal serves as the vitamin, whereas ω-methylpyridoxamine is most effective when pyridoxamine and especially pyridoxamine phosphate are present. The basis for this specificity is not known. It indicates, however, that the effects of the analogues are not entirely explained by their effects on a single pyridoxal phosphate-activated enzyme. Here again, the process being inhibited is not known. If the synthesis of L-alanine is the process inhibited, this must not occur via the glutamic-alanine transaminase, for, as shown below, this enzyme is activated by ω-methylpyridoxal phosphate.

Comparative Effects of Pyridoxal Phosphate and ω-Methylpyridoxal Phosphate on Cell-Free Enzymes—Available evidence (2) indicates that the α-

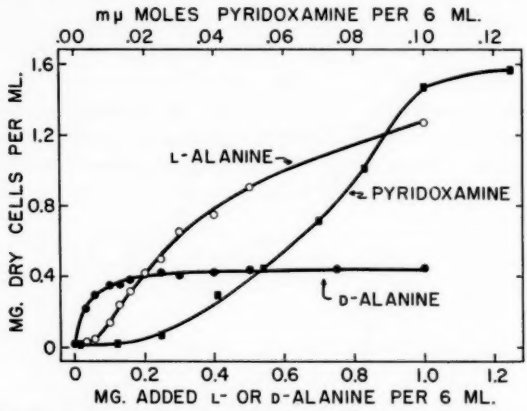


FIG. 3. Comparative activities of L-alanine, D-alanine, and pyridoxamine in promoting growth in a low alanine medium (100 γ of DL-alanine per 6 ml.). 16 hours incubation.

TABLE III

Inhibition of Growth of S. faecalis in Medium Low in L-Alanine by ω-Methyl Analogues of Vitamin B_6

Inhibitor	Vitamin	Inhibition index*
ω-Methylpyridoxal	Pyridoxal	600
	Pyridoxamine	4,000
	" phosphate	1,000
ω-Methylpyridoxamine	Pyridoxal	>19,000
	Pyridoxamine	250
	" phosphate	44
" phosphate	Pyridoxal	>79,000
	Pyridoxamine	4,400
	" phosphate	950

* Ratio of analogue to vitamin that reduces growth from maximal to half maximal. 200 γ of L-alanine per 6 ml. of medium. Incubation time, 16 to 19 hours. Maximal growth corresponds in all cases to approximately 1.4 mg. of cells per ml.

keto acids active in supporting growth of *S. faecalis* are transformed to the corresponding amino acids by transamination with glutamic acid. Since ω-methylpyridoxal supports growth when α-ketoisocaproic, phenylpyruvic, or *p*-hydroxyphenylpyruvic acid replaces the corresponding amino acid, ω-methylpyridoxal phosphate should activate the corresponding transami-

nases. This proved to be the case. Lineweaver-Burk plots showing activation of the leucine-glutamic acid apotransaminase by the two coenzymes are presented in Fig. 4. The values for the dissociation constant, K_{Co}, calculated from these data are 7.2×10^{-7} for pyridoxal phosphate and 7.5×10^{-6} for ω-methylpyridoxal phosphate. Additional determinations gave values of 1.3 and 1.6×10^{-6} for pyridoxal phosphate and 1.1 and 2.5×10^{-5} for ω-methylpyridoxal phosphate. The reasons for the variability in the value from one determination to another are not known; in all cases, however, the affinity of the apotransaminase for ω-methylpyridoxal phosphate is about 0.1 that for pyridoxal phosphate.

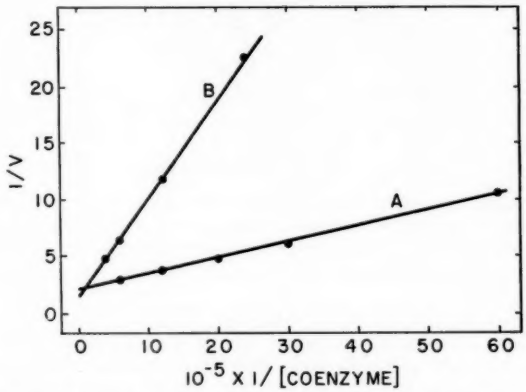


FIG. 4. Comparative activation of the leucine-glutamic acid transaminase by pyridoxal phosphate (Curve A) and by ω-methylpyridoxal phosphate (Curve B). V = the fraction of α-ketoisocaproic acid formed in 30 minutes.

Both the phenylalanine-glutamic acid transaminase (Fig. 5, *A*) and the tyrosine-glutamic acid transaminase (Fig. 5, *B*) are activated by ω-methylpyridoxal phosphate. The similarity in the K_{Co} values of the two apoenzymes for pyridoxal phosphate (9.0×10^{-6} and 7.1×10^{-6}, respectively) and for ω-methylpyridoxal phosphate (8.8×10^{-6} and 5.6×10^{-6}, respectively) indicates that the same enzyme may carry out the transamination of both substrates. In these trials, the affinity of the apoenzyme for the analogue coenzyme is 80 to 90 per cent that for the true coenzyme. However, in both cases the maximal rate of the reaction obtained with the ω-methylpyridoxal phosphate-activated enzyme was less than that obtained with the pyridoxal phosphate-activated enzyme. As already shown for the alanine racemase (1), this may reflect a decreased affinity of the ω-methylpyridoxal phosphate-activated holoenzyme for the substrate. If this were true, the activity of ω-methylpyridoxal relative to that of pyri-

doxal in promoting growth of *S. faecalis* in a medium lacking only tyrosine should increase as the concentration of keto acid in the medium is increased. That such an increase occurs is shown in Table IV.

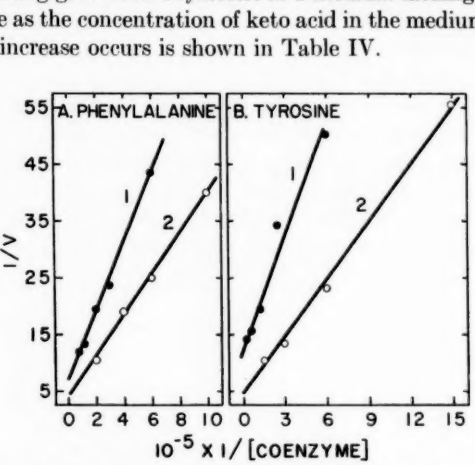


FIG. 5. Comparative activation of phenylalanine-glutamic acid transaminase (*A*) and tyrosine-glutamic acid transaminase (*B*) by ω-methylpyridoxal phosphate (Curve 1) and by pyridoxal phosphate (Curve 2). *V* = the fraction of α-ketoglutaric acid obtained in 30 minutes (see the text).

TABLE IV

Effect of Concentration of p-Hydroxyphenylpyruvic Acid on Requirement of S. faecalis for Pyridoxal and ω-Methylpyridoxal in Tyrosine-Free Medium

Keto acid*	Pyridoxal required for half maximal growth	Relative activity	ω-Methylpyridoxal required for half maximal growth	Relative activity
mg. per 6 ml.	*mμmole per 6 ml.*		*mμmoles per 6 ml.*	
1	0.070	27	5.3	0.36
2	0.023	83	0.87	2.2
3	0.019	100	0.48	4.0

* Approximately the same maximal amount of growth was obtained at all concentrations of *p*-hydroxyphenylpyruvic acid, provided sufficient vitamin B_6 was added.

The alanine-glutamic acid transaminase of *S. faecalis* also is activated by ω-methylpyridoxal phosphate. No quantitative comparisons of the affinity of the apoenzyme for the two coenzymes were carried out. With pyruvate and glutamate as substrates, the fraction of α-ketoglutarate formed in 30 minutes was as follows: no coenzyme, 0.04; pyridoxal phosphate (4.6×10^{-5} M), 0.14; ω-methylpyridoxal phosphate (4.6×10^{-4} M), 0.12.

Finally, the comparative activities of the two coenzymes in activation

of the cysteine apodesulfhydrase of the cells were determined. In preliminary experiments, a K_{Co} value for pyridoxal phosphate of approximately 8×10^{-7} was established and ω-methylpyridoxal phosphate appeared almost inactive. At concentrations 312 times this high, the latter compound activates the enzyme only weakly (Table V). If the activity of this enzyme were required for growth and were in some way made growth-limiting, it is evident that ω-methylpyridoxal would be either inactive or active only at extremely high levels in supporting growth.

TABLE V

Activation of Cysteine Desulfhydrase of S. faecalis by Pyridoxal Phosphate or ω-Methylpyridoxal Phosphate

Supplements to reaction vessel			Pyruvate formed*
Pyridoxal phosphate	ω-Methylpyridoxal phosphate	L-Cysteine	
μmole	*μmole*	*μmoles*	*μmole per ml.*
0	0	2.0	0.107
0.1†	0	2.0	0.672
0	0.25	2.0	0.275

* Pyruvate formed in presence of either coenzyme alone with no substrate was 0.06 to 0.07 μmole per ml.

† This is 125 times the concentration (8.0×10^{-7} M) required for half maximal activation by pyridoxal phosphate.

DISCUSSION

In Table VI the comparative activities of the most active form of vitamin B_6 and its ω-methyl analogue in promoting growth under various conditions discussed here are summarized, together with the comparative activities (as indicated by their K_{Co} values) of pyridoxal phosphate and ω-methylpyridoxal phosphate for the enzymes whose activities are presumed to limit growth under these same conditions. Qualitative correlation is excellent, in that each of the enzymes, as required by the fact that the ω-methyl analogues promote growth, is activated by the analogue coenzyme. An approximate quantitative correlation exists between the growth requirements for vitamin and analogue in the absence of D-alanine and the requirements of the alanine racemase for activity, as discussed elsewhere (1). However, in the remaining instances, such quantitative correlation is not present. ω-Methylpyridoxamine phosphate is more active in promoting growth in the absence of leucine than would be predicted from the ability of ω-methylpyridoxal phosphate to activate the leucine-glutamic acid transaminase; it is considerably less active in promoting growth in the absence of phenylalanine and tyrosine than would be indi-

cated from the effectiveness of the phosphate in activating the corresponding transaminase.

Some possible reasons for this lack of quantitative correlation may be pointed out. Prior to activation of the various enzymes, vitamin B_6 or its active analogues must first be absorbed from the medium, then transformed to the coenzyme. As noted earlier, the variations in the amounts of the various forms of vitamin B_6 (pyridoxal, pyridoxamine, pyridoxamine phosphate) required for activation of any one process (*e.g.* L-leucine synthesis, Table I) point to differences in the amounts absorbed, to differ-

TABLE VI

*Comparative Growth and Enzyme-Activating Activities of Vitamin B_6 and Its ω-Methyl Analogues in S. faecalis**

Growth promotion				Enzyme activation	
Growth limited by synthesis of	PMP required for half maximal growth, mμmole per 6 ml.	Ratio of activities,† PMP:ω-MePMP	Growth-limiting enzyme	K_{Co} for PLP $\times 10^6$	Ratio of activities,‡ PLP:ω-MePLP
D-Alanine	0.0024	1:11–16	Alanine racemase	0.44	1:10
L-Leucine	0.009	1:4.6	Leucine-glutamic acid transaminase	1.2	1:10
L-Phenylalanine	0.008	1:11	Phenylalanine-glutamic acid transaminase	9.0	1:1
L-Tyrosine	0.020	1:7.3	Tyrosine-glutamic acid transaminase	7.1	1:1.3

* The abbreviations used are pyridoxamine phosphate, PMP; ω-methylpyridoxamine phosphate, ω-MePMP; pyridoxal phosphate, PLP; ω-methylpyridoxal phosphate, ω-MePLP.

† Comparison made from amounts required for half maximal growth.

‡ Ratio of K_{Co} value for PLP to that for ω-MePLP.

ences in the ease of conversion to the coenzyme forms, or both. Thus the concentrations of the compounds added to the external medium do not necessarily reflect accurately the concentrations of the coenzymes available within the cell for activation of the various apoenzymes. Just as the efficiency of activation of the various apoenzymes by ω-methylpyridoxal phosphate is less than that by pyridoxal phosphate in all cases tested, so the efficiency of conversion of the ω-methyl analogues within the cell to ω-methylpyridoxal phosphate may be less than that of the naturally occurring vitamin forms; if so, an upper limit to the potency of the analogues as compared to the vitamins would be set by this process, despite the fact that certain of the apoenzymes of the cell are activated almost equally well by coenzyme and analogue coenzyme (see the phenylalanine transaminase,

Table VI). Both in the cell and in the cell-free extracts, several apoenzymes (and perhaps other proteins) that combine with the coenzyme are present. Some of these may be more favorably situated in the highly organized cell for activation by limited amounts of coenzyme than are others; furthermore, since activation by pyridoxal phosphate is a time reaction and not freely reversible (1, 13), relative rates of activation of different apoenzymes also will play a rôle in the growing cell supplied with limited vitamin, but a lesser rôle under the artificial conditions of the experiments *in vitro*. Finally, the lowered affinity of the analogue-activated holoenzymes for their substrates demonstrated for the alanine racemase (1) and likely for the remaining enzymes (see the tyrosine-glutamic acid transaminase, Fig. 5 and Table IV) plays a rôle, for concentrations of the enzyme substrates in the nutrient medium that are sufficient for the vitamin to show its maximal growth-promoting activity may be insufficient for the vitamin analogue to do so.[3]

These several considerations make it clear that quantitative as well as qualitative agreement between the results of studies with growing systems and the cell-free, growth-limiting enzyme may be the exception rather than the rule. It is significant that the best correlation obtained in these studies was found in the case of the alanine racemase (1), in which the affinity of the enzyme for its coenzyme was high and the vitamin requirement for growth correspondingly low.

The cell-free enzyme data demonstrate that there is not only variation in the affinity of each apoenzyme for the same coenzyme, but also an even more extreme variation (from approximately equal affinities for phenylalanine-glutamic acid transaminase to over a 300-fold difference for the cysteine desulfhydrase) with respect to the comparative affinity of a single apoenzyme for pyridoxal phosphate and ω-methylpyridoxal phosphate. The great variation in the latter case emphasizes the importance of unidentified variations in protein structure in determining the ability to combine with the coenzyme, and probably of the resulting holoenzyme to combine with its substrate. In view of the chemical similarity of the two coenzymes studied here, steric factors must play an important rôle in determining these relative affinities.

SUMMARY

ω-Methylpyridoxal, ω-methylpyridoxamine, and ω-methylpyridoxamine phosphate supported growth of *Streptococcus faecalis* in place of vitamin B_6 under conditions that required synthesis of D-alanine (1), or the L isomers

[3] This phenomenon if sufficiently pronounced could readily permit a vitamin analogue that supported growth in the presence of an excess of the substrate to act as a growth inhibitor in the presence of limited substrate, sufficient to permit growth in the presence of the true vitamin.

of leucine, isoleucine, valine, phenylalanine, tyrosine, methionine, or serine. Their activity varied, depending upon the missing amino acid, from about 4 per cent to as high as 25 per cent that of the corresponding form of vitamin B_6. Vitamin B_6 permits growth of *S. faecalis* in the absence of L-cysteine; the ω-methyl analogues do not, but inhibit growth by interfering with utilization of vitamin B_6 under these conditions. They also inhibit growth when availability of L-alanine is limited, and this inhibition is competitively alleviated by vitamin B_6. Thus, depending upon the enzymatic process for which vitamin B_6 is required, these compounds may function in place of it, thus promoting growth, or may interefere with its utilization, thus inhibiting growth.

Synthesis of phenylalanine, tyrosine, and leucine from the corresponding keto acids by the growing organism is presumed to occur through transamination with glutamic acid. As expected from the growth results, the corresponding cell-free transaminases were activated both by pyridoxal phosphate and by ω-methylpyridoxal phosphate; the affinity for the two coenzymes was determined and varied considerably from one enzyme to another. The comparative effectiveness of pyridoxal phosphate and ω-methylpyridoxal phosphate in activating the individual apoenzymes did not correspond to the comparative activities of vitamin B_6 and its ω-methyl analogue in promoting growth under conditions such that activity of these enzymes limited growth. The reasons for these quantitative discrepancies are discussed; it is concluded that quantitative agreement in this system would be the exception rather than the rule.

ω-Methylpyridoxal phosphate also replaces pyridoxal phosphate in activating the alanine-glutamic acid apotransaminase. It was almost inactive as an activator for cysteine desulfhydrase. Indirect evidence indicates that the affinity of the tyrosine-glutamic acid transaminase for its substrate, like that of the alanine racemase (1), is decreased when ω-methylpyridoxal phosphate replaces pyridoxal phosphate as coenzyme.

BIBLIOGRAPHY

1. Olivard, J., and Snell, E. E., *J. Biol. Chem.*, **213,** 203 (1955).
2. Holden, J. T., Wildman, R. B., and Snell, E. E., *J. Biol. Chem.*, **191,** 559 (1951).
3. Ramage, G. R., and Simonsen, J. T., *J. Chem. Soc.*, 532 (1935).
4. Price, V. E., and Levintow, L., *Biochem. Preparations*, **2,** 22 (1952).
5. Meister, A., *Biochem. Preparations*, **3,** 66 (1953).
6. Metzler, D. E., Olivard, J., and Snell, E. E., *J. Am. Chem. Soc.*, **76,** 644 (1954).
7. Heidelberger, C., *Biochem. Preparations*, **3,** 59 (1953).
8. Herbst, R. M., and Shemin, D., *Org. Syntheses*, coll. **2,** 1, 11, 519 (1943).
9. Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, **161,** 311 (1945).
10. Wood, W. A., and Gunsalus, I. C., *J. Biol. Chem.*, **190,** 403 (1951).
11. Metzler, D. E., and Snell, E. E., *J. Am. Chem. Soc.*, **74,** 979 (1952).
12. Metzler, D. E., and Snell, E. E., *J. Biol. Chem.*, **198,** 353 (1952).
13. Meister, A., Sober, H. A., and Peterson, E. A., *J. Biol. Chem.*, **206,** 89 (1954).

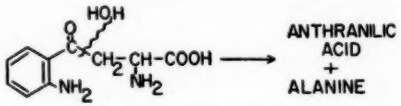
A POSSIBLE MECHANISM FOR KYNURENINASE ACTION

By J. B. LONGENECKER and ESMOND E. SNELL

(From the Biochemical Institute and the Department of Chemistry, The University of Texas, and the Clayton Foundation for Research, Austin, Texas)

(Received for publication, August 12, 1954)

The cleavage of kynurenine to anthranilic acid and alanine is catalyzed by a single pyridoxal phosphate-dependent enzyme, kynureninase, and does not involve a preliminary transamination reaction (1–3). Apparently, the same or a closely related enzyme cleaves 3-hydroxykynurenine to 3-hydroxyanthranilic acid and alanine (3).



These reactions can be formulated as occurring by a simple cleavage reaction involving addition of the elements of water between the β- and γ-carbon atoms of the substrate. Such a formulation is difficult to reconcile with the general mechanism recently proposed from this laboratory (4) or the similar mechanism proposed independently from Braunstein's laboratory (5) for pyridoxal-catalyzed reactions.

An alternative scheme involving an α,β elimination reaction can be formulated in which kynureninase would fall into the same category as serine dehydrase, tryptophanase, and cysteine desulfhydrase (Fig. 1). In this scheme, the Schiff base (I) formed between the pyridoxal phosphate enzyme and kynurenine eliminates a proton from the α-carbon and the anion of the *o*-aminobenzoyl radical (II) from the β-carbon, yielding the Schiff base of α-aminoacrylic acid (III). Intermediate II (as its hydrate) either before or after stabilization as *o*-aminobenzaldehyde then undergoes an oxidation-reduction reaction with III to yield anthranilic acid and the Schiff base of alanine, which hydrolyzes to alanine with regeneration of the phosphopyridoxal enzyme. This scheme is similar to that proposed by Braunstein and Shemyakin (5), but differs from it in proposing that β elimination occurs before rather than after the oxidation-reduction step, thus making unnecessary the assumption that this reaction differs in principle from cleavage of other β substituted amino acids (*e.g.* serine, cysteine, tryptophan, cystathionine).

Intermediate III is the same as that formulated (4) as arising in serine

dehydration, tryptophan cleavage, and similar reactions, in which it hydrolyzes to yield pyridoxal, pyruvate, and ammonia. Support for the feasibility of the proposed mechanism (Fig. 1) would appear if reduction of intermediate III, formed in one of these latter reactions, by *o*-aminobenzaldehyde or other reductant could be demonstrated. It is shown below that, in accordance with the proposal of Fig. 1, alanine and anthranilic acid appear in increased amounts when serine, pyridoxal, and metal salts (used as a source of III) are heated with *o*-aminobenzaldehyde. A much greater

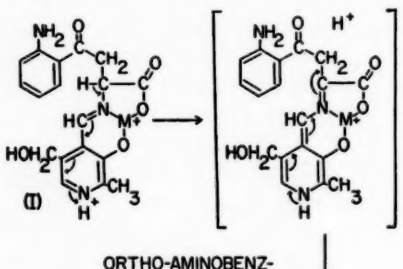


FIG. 1. A possible mechanism for the cleavage of kynurenine by kynureninase. For simplicity, the reactions are formulated with pyridoxal in place of a phosphopyridoxal enzyme.

formation of alanine occurs when thioglycolate replaces *o*-aminobenzaldehyde as the reducing agent.

EXPERIMENTAL

Analytical Techniques—The general technique and certain of the analytical procedures have been described (4, 6, 7). *o*-Aminobenzaldehyde was prepared by reduction of *o*-nitrobenzaldehyde (8). L-Kynurenine sulfate, isolated from the urine of tryptophan-fed rabbits, and kynurenic acid were gifts from Dr. R. P. Wagner.

Pyridoxamine, alanine, and kynurenine were located on paper chromatograms with ninhydrin; anthranilic acid, kynurenic acid, and pyridoxamine by their characteristic fluorescence under ultraviolet light.

Optimal separation on paper chromatograms of the various products from interfering materials was effected with the following solvents and procedures: for alanine and kynurenic acid, water-saturated phenol and the descending technique; for anthranilic acid and pyridoxamine, butanol-acetic acid-water (80:20:20 parts by volume) and the ascending technique.

For quantitative determination, alanine was separated from other components of the reaction mixtures by passage through a 1 × 40 cm. column of Dowex 50 (300 to 500 mesh, H^+ form) and elution with 1.5 N hydrochloric acid. Preliminary test runs established that quantitative recoveries of added alanine were obtained by collecting the acid effluent fraction from 60 to 120 ml. This fraction was evaporated to dryness, dissolved in water and assayed for alanine with *Leuconostoc citrovorum* 8081 (9, 10).

Trial runs established that quantitative extraction of anthranilic acid from the reaction mixtures was effected by extracting twice with an equal volume of ethyl ether. Anthranilic acid in the extract was determined microbiologically with *Lactobacillus arabinosus* 8014, which utilizes anthranilic acid in place of tryptophan (11). Spectrophotometric determinations of this substance failed because of interfering amounts of *o*-aminobenzaldehyde; the latter substance in the amounts present neither increases nor inhibits the response of *L. arabinosus* to anthranilic acid.

Kynurenic acid and pyridoxamine were separated from reaction mixtures by paper chromatography, followed by elution of the appropriate zones into 0.05 M phosphate buffer, and spectrophotometric estimation at 332 and 324 mμ, respectively.

Pyridoxal-Catalyzed Formation of Alanine and Anthranilic Acid from Serine and o-Aminobenzaldehyde—Buffered reaction mixtures (Table I) containing *o*-aminobenzaldehyde, serine, pyridoxal, and potassium alum were heated at 100° for 30 minutes. Appropriate analyses (Table I) showed a considerably enhanced alanine production in the presence of *o*-aminobenzaldehyde at pH 3.5 and 4.0, but not at pH 5.0 and 6.0. The alanine formed in the absence of *o*-aminobenzaldehyde undoubtedly arises in part by transamination of pyruvate (formed from serine via the dehydration reaction under these conditions (12)) with pyridoxamine (formed by transamination of pyridoxal with serine under these conditions (12)). The pH optimum for this alum-catalyzed transamination reaction is near 5.0 (7). Additional alanine may arise in the absence of *o*-aminobenzaldehyde by reduction of III (Fig. 1) by formaldehyde formed under these conditions by the pyridoxal-catalyzed cleavage of serine to formaldehyde and glycine (6).

Corresponding to the "extra" production of alanine at pH 3.5 and 4.0 induced by the presence of *o*-aminobenzaldehyde is an increased production of anthranilic acid (Table I). In the absence of side reactions, the amounts of alanine and anthranilic acid shown in the fourth and seventh columns

should be equimolar; experimentally, anthranilic acid is found in much lower amounts. Separate tests showed, however, that added anthranilic acid was partially destroyed under the reaction conditions. For example, 10 γ of added anthranilic acid were recovered quantitatively from the unheated reaction mixture, pH 3.5. After 30 minutes of heating, only 8 γ (in place of the 14.1 γ expected from that added and that formed (Table I)) were found.

Similar results were obtained repeatedly in both qualitative and quantitative tests, and pyridoxal was required for the reaction. Thus, the ana-

TABLE I

Formation of Alanine and Anthranilic Acid in Reaction Mixtures Containing o-Aminobenzaldehyde, Serine, Pyridoxal, and Metal Ions

Values in micrograms per ml.

pH*	Compound omitted from reaction mixture†		Formed by reduction with *o*-aminobenzaldehyde	Compound omitted from reaction mixture†		Formed by reaction with serine
	None	*o*-Aminobenzaldehyde		None	Serine	
	Alanine formed			Anthranilic acid formed		
3.5	23	13	10	4.1	2.7	1.4
4.0	18	12	6	4.9	3.4	1.5
5.0	16	16	0	5.0	4.6	0.4
6.0	8	9	0	4.1	3.7	0.4

* Formate buffer with ionic strength of 0.1 was used for pH 3.5 and acetate buffer with ionic strength of 0.1 for all other pH values.

† The reaction mixtures 0.04 M in *o*-aminobenzaldehyde, 0.02 M in serine, 0.01 M in pyridoxal, and 0.002 M in $KAl(SO_4)_2 \cdot H_2O$ at the indicated pH values were heated at 100° for 30 minutes.

lytical values support the supposition that reduction of intermediate III (Fig. 1) can occur at the expense of *o*-aminobenzaldehyde, which is thereby oxidized to the anthranilic acid, and argue in favor of the proposed mechanism of Fig. 1 for kynureninase action. The low yield of products under these conditions is to be expected from the many other reactions that serine undergoes under these conditions and the transitory existence of intermediate III. At an enzyme surface, where II and III would coexist in close juxtaposition at the moment of their formation in a specifically directed reaction, no such impediments to the oxidation-reduction would exist.

Pyridoxal-Catalyzed Formation of Alanine from Serine and Sodium Thioglycolate—As added evidence that intermediate III (Fig. 1) can be reduced,

and to throw additional light on the observed formation of alanine from cysteine by a cysteine desulfhydrase in the presence of excess substrate (13), sodium thioglycolate was substituted for *o*-aminobenzaldehyde as the reducing agent in the above reaction mixtures. The results (Table II) indicate that thioglycolate is more efficient than *o*-aminobenzaldehyde in reduction of intermediate III to yield alanine. Significantly, the pH optimum for the reduction is the same in the two cases. Again, pyridoxal and alum were required for the reaction. The result confirms the mechanism suggested for alanine production from cysteine by Ohigashi *et al.* (13) with the modification that the Schiff base of cysteine with pyridoxal (or the

TABLE II

Production of Alanine by Reaction of Serine with Sodium Thioglycolate in Presence of Pyridoxal and Alum

pH*	Compound omitted from reaction mixture†		Formed by reduction with sodium thioglycolate
	None	Sodium thioglycolate	
	Alanine formed, γ per ml.		
3.5	62	13	49
4.0	52	12	40
5.0	39	16	23
6.0	19	9	10

* The same buffers listed in Table I were used in these reaction mixtures.

† The reaction mixtures 0.04 M in sodium thioglycolate, 0.02 M in serine, 0.01 M in pyridoxal, and 0.002 M in $KAl(SO_4)_2 \cdot H_2O$ at the indicated pH values were heated at 100° for 30 minutes.

pyridoxal phosphate-activated cysteine desulfhydrase) replaces the free amino acid. By providing additional evidence for the type of oxidation-reductions indicated in Fig. 1, the result also supports the proposed mechanism for kynureninase action. It may be noted here that intermediate III has also been "trapped" by reaction with indole to form tryptophan in a pyridoxal-catalyzed reaction (4).

Reaction of Kynurenine with Pyridoxal in Presence of Alum—It was hoped that a direct demonstration of the reactions of Fig. 1 might be achieved in model systems containing kynurenine, pyridoxal, and metal ions. However, in the limited number of experiments permitted by supplies of kynurenine, neither alanine nor anthranilic acid could be detected in reaction mixtures heated at pH 3.5 or 5.0. Instead, kynurenic acid, presumably formed by spontaneous ring closure of *o*-aminobenzoylpyruvic acid produced from kynurenine by transamination with pyridoxal, was formed in

equimolar amount with pyridoxamine. For example, a reaction mixture 0.04 M in kynurenine, 0.01 M in pyridoxal, 0.002 M in potassium alum, and containing acetate buffer of 0.1 ionic strength, pH 5.0, was heated at 100° for 30 minutes. Analysis of the mixture showed that 10 per cent of the kynurenine had been transformed to kynurenic acid, with formation of an equimolar amount (3.9 μmoles per ml.) of pyridoxamine. Either the kynureninase reaction does not occur in model systems under these conditions, or it occurs relatively slowly.

SUMMARY

The feasibility of a mechanism for the splitting of kynurenine by kynureninase that permits visualization of this reaction in terms of the general mechanism previously proposed (4) for vitamin B_6-catalyzed reactions was tested in model reactions. This mechanism involves an α,β elimination of the elements of *o*-aminobenzaldehyde from the Schiff base of kynurenine with kynureninase, with the formation of a Schiff base of the pyridoxal phosphate enzyme with α-aminoacrylic acid. Oxidation-reduction on the enzyme surface between the latter Schiff base and *o*-aminobenzaldehyde, followed by hydrolysis, yields alanine and anthranilic acid.

The proposed Schiff base of α-aminoacrylic acid and pyridoxal was generated in model systems by heating serine with pyridoxal and aluminum ions. It was shown that alanine formation under these conditions was enhanced by addition of *o*-aminobenzaldehyde, with the simultaneous formation of anthranilic acid. Substitution of sodium thioglycolate for *o*-aminobenzaldehyde increased alanine formation even more, thus emphasizing the possibility of the proposed oxidation-reduction step in kynureninase action and providing experimental evidence in support of the proposed explanation (4) for the observed (13) formation of alanine during enzymatic desulfhydration of cysteine.

When kynurenine, pyridoxal, and aluminum ions are heated at pH 3.5 or 5.0, kynurenic acid and pyridoxamine are formed in equimolar amounts. Kynurenic acid must arise by spontaneous ring closure of *o*-aminobenzoylpyruvic acid formed by transamination between kynurenine and pyridoxal.

BIBLIOGRAPHY

1. Braunstein, A. E., Goryachenkova, E. V., and Paskhina, T. S., *Biokhimiya*, **14**, 163 (1949).
2. Miller, I. L., and Adelberg, E. A., *J. Biol. Chem.*, **205**, 691 (1953).
3. Jakoby, W. B., and Bonner, D. M., *J. Biol. Chem.*, **205**, 699 (1953).
4. Metzler, D. E., Ikawa, M., and Snell, E. E., *J. Am. Chem. Soc.*, **76**, 648 (1954).
5. Braunstein, A. E., and Shemyakin, M. M., *Biokhimiya*, **18**, 393 (1953).
6. Metzler, D. E., Longenecker, J. B., and Snell, E. E., *J. Am. Chem. Soc.*, **76**, 639 (1954).

7. Metzler, D. E., and Snell, E. E., *J. Am. Chem. Soc.*, **74,** 979 (1952).
8. Smith, L. I., and Opie, J. W., *Org. Syntheses*, **28,** 11 (1948).
9. Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Biol. Chem.*, **177,** 533 (1949).
10. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, **177,** 545 (1949).
11. Snell, E. E., *Arch. Biochem.*, **2,** 389 (1943).
12. Metzler, D. E., and Snell, E. E., *J. Biol. Chem.*, **198,** 353 (1952).
13. Ohigashi, K., Tsunetoshi, A., Uchida, M., and Ichihara, I., *J. Biochem.*, Japan, **39,** 211 (1952).

By

(*From

Th
aluro
mainl
and e
glucu
tures
curon
of the
strate
while
Helfe
glucu
two e
gosac
acety
glucu

Ar
color
sugar
activ
(7).
In
aceti

* T
and t
this
Socie
† T
the r
Colu
‡

XUM

ENZYMATIC FORMATION OF MONOSACCHARIDES FROM HYALURONATE*

By ALFRED LINKER,† KARL MEYER, AND BERNARD WEISSMANN‡

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Edward Daniels Faulkner Arthritis Clinic, Presbyterian Hospital, New York, New York)

(Received for publication, September 8, 1954)

The products of hydrolysis of hyaluronate by purified testicular hyaluronidase were shown to consist of mixtures of oligosaccharides composed mainly of tetra-, hexa-, and octasaccharides in ratios depending on time and enzyme concentration (2). Crude testicular or liver extracts liberated glucuronic acid and *N*-acetylglucosamine from such oligosaccharide mixtures. The liberation of monosaccharides appeared to parallel the β-glucuronidase activity of these extracts (3). In this report the mechanism of the monosaccharide formation is further investigated. It will be demonstrated that β-glucuronidase action leads to liberation of D-glucuronic acid, while a hexosaminidase, similar to the β-glucosaminidase first described by Helferich and Iloff (4), yields *N*-acetylglucosamine. The hydrolysis of the glucuronidic and hexosaminidic bonds is a two-step reaction in which the two enzymes alternately attack the terminal (non-reducing) ends of the oligosaccharides. The end-products of these reactions are glucuronic acid, *N*-acetylglucosamine, and *N*-acetylhyalobiuronic acid (5) which contains a β-glucuronidic bond resistant to β-glucuronidase.

EXPERIMENTAL

Analytical methods for total hexosamine, acetylglucosamine end-group color, colorimetric uronic acid (Dische), uronic acid CO_2, and reducing sugar (ferricyanide) have been reported previously (6). β-Glucuronidase activity was measured by the hydrolysis of phenolphthalein glucuronide (7).

In the paper chromatograms (Whatman No. 1 filter paper), a butanol-acetic acid-water mixture with downward irrigation was employed, as de-

* This work has been supported by grants from the National Institutes of Health and the New York Chapter of the Arthritis and Rheumatism Foundation. Part of this work has been reported before the Forty-third annual meeting of the American Society of Biological Chemists at New York in 1952 (1).

† Taken in part from a thesis submitted by Alfred Linker in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Faculty of Pure Science, Columbia University.

‡ Present address, Department of Medicine, Mount Sinai Hospital, New York.

XUM

scribed earlier (2). Ehrlich's reagent was used to detect compounds having an amino sugar on the free reducing end (8). A benzidine reagent (9) and an aniline reagent (10) were used to detect free uronic acid.

All enzyme experiments were run in 0.1 M acetate buffer,-0.15 M in NaCl, at pH 5.0, except where noted otherwise.

The preparation of hyaluronic acid and a hyaluronate oligosaccharide mixture, obtained from the polymer by 24 hour digestion with testis hyaluronidase, was described previously (3).

Isolated oligosaccharides were obtained by ion exchange chromatography of a testicular hyaluronidase digest of hyaluronate (2).

N-Acetyl-β-phenyl-D-glucosaminide $[\alpha]_D$ $-5°$ was prepared according to Helferich and Iloff (4). The α anomer, $[\alpha]_D$ $+208°$, was prepared according to Zechmeister and Toth by the ion exchange step recommended by Roseman and Dorfman (11).

The preparation of crude liver glucuronidase (MIV-94, MV-13), purified liver glucuronidase (W-I-22),[1] crude testis enzyme (F-XIV) (3), and of pneumococcus enzyme (MIV-86) was described previously (13). A β-glucosaminidase was prepared from sheep livers by a modification of a method by Bernfeld and Fishman for the preparation of β-glucuronidase from calf spleen (14). Four fractions were obtained of low glucuronidase but considerable β-glucosaminidase activity. The α-glucosaminidase activity in all fractions was less than 0.5 unit per mg. The fractions had been assayed for α- and β-glucosaminidase activity by the following modification of the method of East, Madinaveitia, and Todd (15).

Reagents—(1) M/6 acetate buffer, pH 4.6; (2) 0.6 per cent solution of the *N*-acetylphenyl-D-glucosaminide; (3) 0.4 M sodium carbonate; (4) Folin-Ciocalteu reagent; (5) standard solution of phenol, 1 mg. per ml.

Procedure—Aliquots of substrate solutions (0.5 ml.) were placed in small test-tubes, and 0.5 ml. of enzyme solution of desired concentration in buffer was added. The tubes were incubated at 37° from $\frac{1}{2}$ to 2 hours, depending on the expected activity. An enzyme blank containing no substrate was included. After the end of the incubation period, 2.0 ml. of Folin reagent were added, the tubes were centrifuged, and 1.0 ml. of the supernatant solution was added to 10.0 ml. of the sodium carbonate solution and mixed at once. After 25 to 45 minutes, the solutions were transferred to cuvettes and read in a spectrophotometer at 650 mu. A reagent blank and phenol standards were also read at the same time. 1 γ of phenol liberated per hour is equal to 1 glucosaminidase unit (see Table I).

Hyaluronate oligosaccharides were incubated for 24 hours in buffer at 10 mg. per ml. with enzymes from different sources and varying degrees of purity. The crude enzymes had a glucuronidase activity of about 300 phe-

[1] None of the liver extracts used contained hyaluronidase demonstrable by turbidimetric or reductimetric methods (12).

nolphthalein units per mg., the purified enzymes about 6600 units per mg.; all were used at equal β-glucuronidase activity. The digests were put on a carbon column, eluted, and the eluates analyzed for acetylglucosamine and glucuronic acid. The results are presented in Table II.

It can be seen that the enzymes, when used at equal β-glucuronidase activity, liberate the same amount of glucuronic acid. The liberation of acetylglucosamine, however, seems to be dependent on another enzyme, presumably a β-glucosaminidase.

TABLE I

Activity of Liver Enzyme Fractions

Fraction	β-Glucosaminidase	β-Glucuronidase
	units per mg.	*units per mg.*
MV-23-I	323	3.0
MV-23-II	209	2.0
MV-23-III	484	19.0
MV-23-IV	157	22.0

TABLE II

Comparison of Enzymes in Terms of Glucuronidase and Glucosaminidase Activity

Enzyme	Phenolphthalein units per ml. incubation mixture	Glucosaminidase units per ml. incubation mixture	Uronic acid*	Acetyl-glucosamine*
			mg.	*mg.*
Crude testis (F-XIV)	350	2200	12.0	7.0
" liver (MIV-94)	350	500	11.0	7.0
Purified liver (W-I-22)	350	9	11.7	<0.5

* Uronic acid and acetylglucosamine, respectively, liberated per 100 mg. of substrate.

Hyaluronate oligosaccharides were incubated for 24 hours at 20 mg. per ml. in buffer with the following enzymes at 500 phenolphthalein units per ml.: purified liver glucuronidase (W-I-22), crude liver enzyme (MIV-94), and crude testis enzyme (F-XIV). For exhaustive hydrolysis, enzyme was added again after 24 hours and after 48 hours; the reaction was stopped after 72 hours. Aliquots containing 0.4 mg. of material were spotted on paper which was developed with butanol-acetic acid-water (50:12:25). A reproduction of the chromatogram is shown in Fig. 1.

A separate chromatogram was run under the same conditions and sprayed with benzidine reagent for detection of free uronic acid. All digests showed the presence of free glucuronic acid.

It can be seen from Fig. 1 that in the 24 hour digests of the crude en-

zymes there were three new spots. Spot I indicated acetylglucosamine; Spot II and Spot III, as will be shown, indicated a disaccharide and a trisaccharide, respectively. The exhaustive purified enzyme digest did not seem to contain acetylglucosamine or disaccharide, as both Spots I and II were absent. The exhaustive digest with crude testis enzyme contained only monosaccharides and a disaccharide. This residual disaccharide was compared by paper chromatography with *N*-acetylhyalobiuronic acid, a

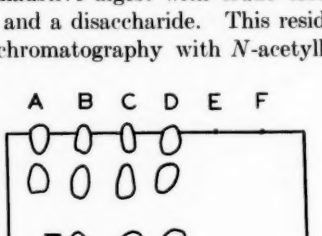


FIG. 1. Reproduction of a paper chromatogram showing the action of enzymes on hyaluronate oligosaccharides. Solvent, butanol-acetic acid-water (50:12:25); 20 hours irrigation. *A*, initial oligosaccharides (control); *B*, 24 hour digest of oligosaccharides with crude liver enzyme; *C*, 24 hour digest with crude testis enzyme; *D*, exhaustive digest with purified liver enzyme; *E*, exhaustive digest with crude testis enzyme; *F*, acetylglucosamine (control). Spot I indicates acetylglucosamine; Spots II and III, the positions of two unknowns, subsequently shown to be a disaccharide and a trisaccharide.

disaccharide of known structure (5). It gave a spot with the same R_F value as *N*-acetylhyalobiuronic acid.

Isolation of Residual Material Corresponding to Spot II—1 gm. of a hyaluronate oligosaccharide mixture and 200 mg. of crude testis enzyme (F-XIV) were dissolved in 100 ml. of buffer and incubated at 37° for 24 hours under toluene. After 24 hours and again after 48 hours, 100 mg. of enzyme were added. The total incubation time was 77 hours. The reaction mixture was put on a carbon column (20 gm. of Darco G-60 and 10 gm. of Celite 535) and eluted with the following solvents: 500 ml. of water, 500 ml. of 5 per cent ethanol, 500 ml. of 15 per cent ethanol, and 700 ml. of 5 per cent pyridine. Fractions of 100 ml. were collected and analyzed for uronic acid and reducing sugar. The water eluate containing mostly free glucuronic acid and the 15 per cent alcohol fraction containing only small

amounts of material were discarded. The 5 per cent alcohol fraction containing mostly free acetylglucosamine, as shown by colorimetric analysis (6) and paper chromatography, was evaporated to a small volume *in vacuo*. Alcohol (95 per cent) and ether were added to turbidity. Three crops of crystals were collected after standing at 4°; the total yield was 138.5 mg.; m.p. 200.5–202.5° (hot stage), unchanged on admixture with authentic *N*-acetyl-D-glucosamine; $[\alpha]_D^{25}$ +43° (*c* 2.0, H_2O). (Previously *N*-acetyl-D-glucosamine had not been isolated from hyaluronic acid.)

The 5 per cent pyridine eluate was evaporated *in vacuo* to a small volume. Paper chromatography of this fraction indicated that it contained for the most part material corresponding to Spot II, together with small amounts of higher molecular weight material. This fraction, after repurification on a carbon column (214 mg.), appeared homogeneous by paper chromatography. The analysis of this material for uronic acid (CO_2), hexosamine, and reducing sugar corresponded closely to that of *N*-acetylhyalobiuronic acid (5); $[\alpha]_D^{24}$ of residual disaccharide −31°, of *N*-acetylhyalobiuronic acid −32°.

The residual disaccharide was further characterized as the crystalline heptaacetyl methyl ester (5); $[\alpha]_D^{25}$ +27° (*c* 2, $CHCl_3$); m.p. 120°. No melting point depression with authentic methyl heptaacetyl hyalobiuronate, m. p. 120°, $[\alpha]_D^{25}$ +24.5°.

This confirmed the identity of the two disaccharides.

Identification of Material Corresponding to Spot III (*Fig. 1*)—Hyaluronate oligosaccharides were incubated with crude liver enzyme, and the 24 hour digest was put on paper at 0.4 mg. per spot, as described above. The material in Spot III was eluted with water and the eluate analyzed for total hexosamine and uronic acid. A ratio of hexosamine to uronic acid of 1.85 was found (theory for a trisaccharide, 1.84).

In order to isolate this trisaccharide in good yield, an oligosaccharide mixture containing a high percentage of tetrasaccharide (2) was prepared. To 1 gm. of this mixture in 100 ml. of buffer, 100 mg. of a liver preparation (MV-13) high in glucuronidase activity (1150 phenolphthalein units per mg.) and low in β-glucosaminidase activity (260 phenol units per mg.) were added, and the solution was incubated for 24 hours at 37°. A precipitate, formed on addition of an equal volume of 95 per cent ethanol, was discarded. The supernatant solution was evaporated *in vacuo* to about 30 ml. and put on a carbon column (20 gm. of Darco G-60 and 10 gm. of Celite 535). The column was eluted with the following solvents: 500 ml. of water, 250 ml. of 5 per cent ethanol, 750 ml. of 15 per cent ethanol, 750 ml. of 0.5 per cent pyridine, and 300 ml. of 5 per cent pyridine. The eluates were analyzed for reducing sugar, uronic acid, and, after evaporation *in vacuo* to small volumes, by paper chromatography. The water and 5 per

cent alcohol fractions containing mostly free uronic acid were discarded. The eluates containing considerable amounts of trisaccharide were put back on a carbon column, eluted with a similar solvent mixture as above, and checked by paper chromatography; those containing only the trisaccharide spot were set aside and the others put back on a column again. All fractions containing only trisaccharide were combined, evaporated *in vacuo* to a small volume, and lyophilized.

The final yield of material corresponding to Spot III was 204 mg. Analysis, hexosamine 55.1 per cent, uronic acid (CO_2) 29.6 per cent, nitrogen (Kjeldahl) 4.52 per cent, reducing sugar (ferricyanide) 54.4 per cent, reducing sugar (hypoiodite) 51.0 per cent; $[\alpha]_D^{23}$ $-16°$ (*c* 1.0, H_2O). Molar ratios, hexosamine-nitrogen 0.95, uronic acid-nitrogen 0.47, hexosamine-uronic acid 1.86. These analyses show that this material is a trisaccharide composed of two amino sugar units and one uronic acid unit.

As may be seen in Fig. 1, the digest obtained by the action of purified glucuronidase contained fractions of higher molecular weight than trisaccharide (Spot III), even after exhaustive digestion. Paper chromatograms of such a digest were run in butanol-acetic acid-water (50:15:35) for 48 hours. It was found that the fractions obtained from the purified glucuronidase digest did not correspond to the original oligosaccharides. Spots were resolved moving at an intermediate rate between the known tetra- and hexasaccharides and between hexa- and octasaccharides. In another run the spot, moving at a somewhat slower rate than a control of known tetrasaccharide, was eluted and analyzed for uronic acid and hexosamine. The ratio of hexosamine to uronic acid was 1.43 (theory for pentasaccharide 1.39). This material thus appears to be a pentasaccharide.

These results indicated that the glucuronidase seemed to have acted on all the oligosaccharides by removing one uronic acid unit from the nonreducing end of the molecule. To clarify this further, experiments were carried out with isolated oligosaccharides.

Action of Glucuronidase and Glucosaminidase on Isolated Oligosaccharides—Hexasaccharide (10 mg.) (2) was incubated at 37° in 1.0 ml. of buffer with 500 phenolphthalein units of purified liver β-glucuronidase (W-I-22). Aliquots containing 0.4 mg. of material were withdrawn at 6 and 23 hours and chromatographed. A reproduction of the chromatogram is shown in Fig. 2. After 48 hours, 3.2 mg. of liver enzyme (MV-23-I), high in glucosaminidase and low in glucuronidase, were added to the digestion mixture. Aliquots containing 0.4 mg. of material were withdrawn and chromatographed (see Fig. 3). Separate chromatograms had to be run for a shorter time and with a solvent lower in water content, butanol-acetic acid-water (50:12:25), in order to show monosaccharides. (For the sake of clarity the monosaccharides when present are reproduced on the same figure.)

It can be seen from Fig. 2 that, after 6 hours hydrolysis by the glucuronidase, two new spots appeared on the chromatogram. The fastest corresponded to glucuronic acid. The second spot, which increased in intensity after 23 hours of hydrolysis, corresponded to the material assumed to be a pentasaccharide. At no time was any tetrasaccharide formed. Fig. 3 shows the results after glucosaminidase had been added. After 7

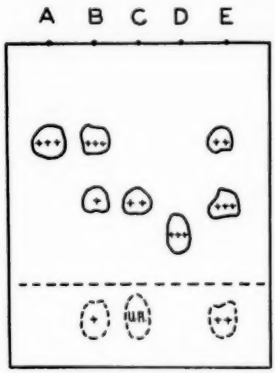


FIG. 2

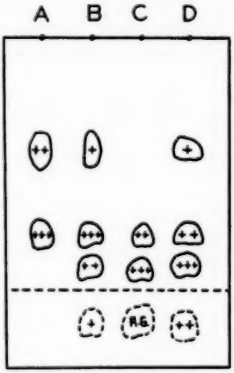


FIG. 3

FIG. 2. Reproduction of a paper chromatogram showing the action of β-glucuronidase on hyaluronate hexasaccharide. Solvent, butanol-acetic acid-water (50:15:35); 48 hours irrigation. *A*, zero time; *B*, 6 hours hydrolysis; *C*, 0.1 mg. of pentasaccharide as control (see the text); *D*, 0.1 mg. of tetrasaccharide (control); *E*, 23 hours hydrolysis. The spots below the dash line were obtained from a chromatogram irrigated for 16 hours. The spots were developed with aniline reagent. *U. A.*, 0.05 mg. of glucuronic acid (control); + indicates the relative intensity of the spots.

FIG. 3. Reproduction of a paper chromatogram showing the action of glucosaminidase on pentasaccharide. Solvent, butanol-acetic acid-water (50:15:35); 48 hours irrigation. *A*, zero time; *B*, 7 hours hydrolysis; *C*, controls; 0.1 mg. of pentasaccharide (slower spot) and 0.1 mg. of tetrasaccharide; *D*, 23 hours hydrolysis. The spots below the dash line were obtained from a chromatogram irrigated for 16 hours. *A. G.*, 0.05 mg. of acetylglucosamine (control); + indicates the relative intensity of the spots.

hours of hydrolysis, and increasingly so after 23 hours, acetylglucosamine and tetrasaccharide had been formed.

Tetrasaccharide (10 mg.) was incubated at 37° in 1.0 ml. of buffer with 500 phenolphthalein units of the purified β-glucuronidase (W-I-22). Aliquots containing 0.4 mg. of material were chromatographed. A reproduction of the chromatogram is given in Fig. 4. After 4 hours of hydrolysis, glucuronic acid and trisaccharide have been formed; both increased after 23 hours. No spots corresponding to disaccharide or acetylglucosamine could be detected.

Trisaccharide (10 mg.), isolated as described above, was incubated at 37° in 1.0 ml. of buffer with 3.0 mg. of the enzyme high in glucosaminidase (MV-23-I). Aliquots containing 0.4 mg. of material were withdrawn at 6 and 23 hours and chromatographed on paper (see Fig. 5). As can be seen, spots corresponding to acetylglucosamine and disaccharide had appeared after 6 and 23 hours of hydrolysis.

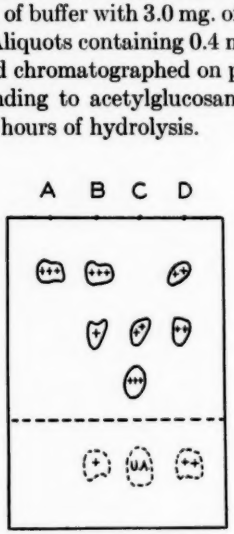


FIG. 4

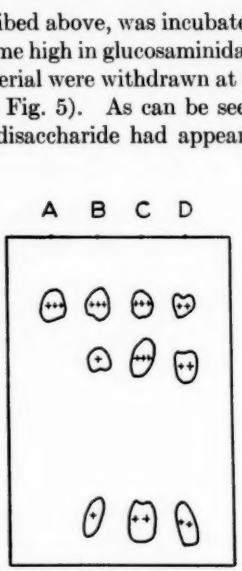


FIG. 5

FIG. 4. Reproduction of a paper chromatogram showing the action of β-glucuronidase on tetrasaccharide. Solvent, butanol-acetic acid-water (50:12:25); 43 hours irrigation. *A*, zero time; *B*, 4 hours hydrolysis; *C*, controls; 0.1 mg. of trisaccharide and 0.1 mg. of disaccharide (faster spot); *D*, 23 hours hydrolysis. The spots below the dash line were obtained from a chromatogram irrigated for 16 hours. The spots were developed with aniline reagent. *U. A.*, 0.05 mg. of glucuronic acid (control); + indicates the relative intensity of the spots.

FIG. 5. Reproduction of a paper chromatogram showing the action of glucosaminidase on trisaccharide. Solvent, butanol-acetic acid-water (50:12:25); 24 hours irrigation. *A*, zero time; *B*, 6 hours hydrolysis; *C*, controls; 0.1 mg. of trisaccharide (slowest spot), 0.1 mg. of disaccharide, and 0.05 mg. of acetylglucosamine (fastest spot); *D*, 23 hours hydrolysis; + indicates the relative intensity of the spots.

In none of the experiments was any evidence found for the presence of oligosaccharides having a free end-group other than acetylglucosamine; all spots detected by the aniline reagent also yielded color with Ehrlich's amino sugar reagent (aside from free glucuronic acid).

These data showed that the glucuronidase is an "exo" enzyme and does not hydrolyze internal linkages.

In order to determine whether the glucosaminidase hydrolyzed internal linkages or was restricted to the hydrolysis of end-groups, the following experiment was carried out. Tetrasaccharide and hexasaccharide, respectively, were incubated at 37° in buffer with 2000 β-glucosaminidase units

of liver enzyme (MV-23-I) high in glucosaminidase. After 16 hours, aliquots containing 0.3 mg. of material were withdrawn and run on a paper chromatogram. No hydrolysis could be observed, indicating that the enzyme did not hydrolyze internal linkages. It is therefore also an "exo" enzyme.

As all the enzymes used contained not only β-glucosaminidase but also α-glucosaminidase, and, as only one of the enzymes was highly purified, it was not certain that the same enzyme acting on the standard substrate (*N*-acetyl phenyl-β-D-glucosaminide) was also responsible for the hydrolysis of the hyaluronate oligosaccharides. The following experiments were carried out to clarify this.

TABLE III

Action of Glucosaminidases on Trisaccharide

Enzyme	β units per mg.	α units per mg.	β units used	α units used	Acetyl-glucosamine
					γ*
MV-13	194	3.2	2000	26	152
MV-23-I	380	0.3	2000	3	117
F-XIV	790	5.5	2000	14	73
MV-23-III	448	<0.2	2000	<1	55
MV-23-IV	143	<0.2	2000	<3	67
MIV-86	800	<0.1	2000	<1	0
Emulsin	125	<0.1	2000	<1	0

* Liberated per mg. of trisaccharide in 8 hours of hydrolysis.

Action of Various Enzymes on Trisaccharide—Enzymes from different sources and of different degrees of purity, containing α- and β-glucosaminidase (11), were compared; they were used at equal concentrations in terms of their β-glucosaminidase activity, and their action on trisaccharide was studied by measuring the amount of acetylglucosamine liberated. Trisaccharide was dissolved at 20 mg. per ml. in buffer containing 2 mg. of gelatin (Knox 6 per cent) per ml. The following enzymes were added so that their final concentration was 2000 β-glucosaminidase units per ml.: MV-13 (crude liver enzyme), MV-23-I, III, IV (liver enzymes), F-XIV (crude testis enzyme), MIV-86 (pneumococcal enzyme), emulsin (Mann Chemical Company). The reaction mixtures were incubated for 8 hours at 37°. Aliquots containing 0.5 mg. of material were put on paper and the chromatogram developed with butanol-acetic acid-water (50:12:25) for 16 hours. Four columns were run for each aliquot, the outside columns were sprayed with Ehrlich's reagent, and a strip was cut out of the center columns at the level of acetylglucosamine; it was eluted with water, and

the eluate made up to 5.0 ml. in a volumetric flask and analyzed for acetylglucosamine. The results of this experiment are presented in Table III. It can be seen that emulsin and bacterial β-glucosaminidase with high activity against the standard substrate do not act on the trisaccharide. There is no correlation between α-glucosaminidase activity and action on the trisaccharide. There is the suggestion of a correlation between β-glucosaminidase activity as measured by the hydrolysis of the standard substrate (*N*-acetyl phenyl-β-D-glucosaminide) and hydrolysis of the trisaccharide.

Action of Crude Enzymes on Chondroitin Sulfate Oligosaccharides—When cartilage chondroitin sulfate is hydrolyzed by testicular hyaluronidase, a mixture of sulfated oligosaccharides is obtained.[2] This mixture was incubated with crude liver and testis enzymes as described for the hyaluronate oligosaccharides. The digest was chromatographed on paper, and the results showed that free glucuronic acid was liberated, but no spot corresponding to acetylhexosamine could be detected. At present it cannot be decided whether the hexosaminidase acts on these fractions, since the mobility on paper of the sulfated acetylchondrosamine is not known.

DISCUSSION

All hyaluronidases thus far investigated hydrolyze only the glucosaminidic bonds of hyaluronate, with the production of even-numbered oligosaccharide fractions with *N*-acetylglucosamine at the reducing end and glucuronic acid at the terminal non-reducing end. The hydrolysis of tetrasaccharide by purified β-glucuronidase yielding glucuronic acid and a trisaccharide, a β-(?)-D-*N*-acetylglucosaminyl-β-D-glucuronyl-β-D-1-3-*N*-acetyl-glucosamine, permits us to conclude that (*a*) in the tetrasaccharide the component carbohydrates alternate and (*b*) the β-glucuronidase attacks only a terminal glucuronidic bond. This is further substantiated by the formation of a pentasaccharide from a hexasaccharide by this enzyme. The hydrolysis of pentasaccharide into tetrasaccharide and *N*-acetylglucosamine and of trisaccharide into *N*-acetylglucosamine and disaccharide by liver and testicular extracts indicates a second enzyme hydrolyzing the terminal glucosaminidic bond. This second enzyme probably is a β-glucosaminidase, although the identification of this enzyme with that hydrolyzing β-phenyl-*N*-acetylglucosaminide has not been established. In fact, the failure of β-glucosaminidase of bacterial and plant origin (emulsin) to hydrolyze the trisaccharide apparently precludes this identification. Extracts containing both glucuronidase and glucosaminidase liberate both monosaccharides by acting alternately on the terminal groups of the oligosaccharide fractions. The residual disaccharide, *N*-acetylhyalobiuronic acid,

[2] Linker and Meyer, unpublished experiments.

is refractory to enzymatic hydrolysis. This disaccharide can be produced by three methods: (1) as a minor product by hydrolysis of hyaluronate with purified testicular hyaluronidase, (2) as a major product by hydrolysis of oligosaccharides with crude testicular or liver extracts, and (3) by *N*-acetylation of hyalobiuronic acid. The three products thus obtained are identical. This leaves little doubt that this disaccharide of established configuration (5) is the main, if not the only, repeating unit of hyaluronic acid. The specificity and biological rôle of the hexosaminidase are not known. The wide distribution of this enzyme, however, might indicate the wide occurrence of compounds having a terminal β-glucosaminidic bond. One important distinction between these enzymes and hyaluronidases is the latter are endohexosaminidases.

It has not been possible to demonstrate the degradation of high polymers of hyaluronic acid from the terminal end analogous to the β-amylase action on starch. However, the possibility of the existence of such a pathway of degradation in the organism cannot be ruled out.

SUMMARY

The production of monosaccharides by extracts of testis and liver from hyaluronic acid digests has been reexamined. Glucuronic acid, as found earlier, is formed by the action of β-glucuronidase present in these extracts. *N*-Acetylglucosamine, crystallized from enzymatic digests for the first time, has been shown to be liberated by a second enzyme presumably a β-glucosaminidase, present with β-glucuronidase. Purified β-glucuronidase hydrolyzes a tetrasaccharide, with the formation of glucuronic acid and a trisaccharide composed of 2 moles of *N*-acetylglucosamine and 1 mole of glucuronic acid. Hexasaccharide is hydrolyzed to glucuronic acid and a pentasaccharide. The glucosaminidase hydrolyzes only the terminal glucosaminidic bonds of the odd-numbered oligosaccharides, produced by β-glucuronidase action, to yield *N*-acetylglucosamine and the next lower even-numbered oligosaccharide. Both enzymes thus act stepwise only on terminal groups. Extracts containing mixtures of the two enzymes thus yield both monosaccharides and a disaccharide, *N*-acetylhyalobiuronic acid, identical with the repeating disaccharide of known structure of hyaluronic acid. The identity of the glucosaminidase with the β-glucosaminidase of earlier authors remains questionable.

BIBLIOGRAPHY

1. Linker, A., and Meyer, K., *Federation Proc.*, **11**, 249 (1952).
2. Weissmann, B., Meyer, K., Sampson, P., and Linker, A., *J. Biol. Chem.*, **208**, 417 (1954).
3. Meyer, K., Linker, A., and Rapport, M. M., *J. Biol. Chem.*, **192**, 275 (1951).
4. Helferich, B., and Iloff, A., *Z. physiol. Chem.*, **221**, 252 (1933).

XUM

5. Weissmann, B., and Meyer, K., *J. Am. Chem. Soc.*, **76,** 1753 (1954).
6. Rapport, M. M., Meyer, K., and Linker, A., *J. Am. Chem. Soc.*, **73,** 2416 (1951).
7. Fishman, W. H., Springer, B., and Brunetti, R., *J. Biol. Chem.*, **173,** 449 (1948).
8. Partridge, S. M., *Biochem. J.*, **42,** 238 (1948).
9. Horrocks, R. M., *Nature*, **164,** 444 (1949).
10. Hough, L., Jones, J. K. N., and Wadman, W. H., *J. Chem. Soc.*, 1702 (1950).
11. Roseman, S., and Dorfman, A., *J. Biol. Chem.*, **191,** 607 (1951).
12. Meyer, K., and Rapport, M. M., *Advances in Enzymol.*, **13,** 199 (1952).
13. Rapport, M. M., Linker, A., and Meyer, K., *J. Biol. Chem.*, **192,** 283 (1951).
14. Bernfeld, P., and Fishman, W. H., *Arch. Biochem.*, **27,** 475 (1950).
15. East, M. E., Madinaveitia, J., and Todd, A. R., *Biochem. J.*, **35,** 872 (1941).

HYDROXYLYSINE: ISOLATION FROM GELATIN AND RESOLUTION OF ITS DIASTEREOISOMERS BY ION EXCHANGE CHROMATOGRAPHY

By PAUL B. HAMILTON and ROBERTA A. ANDERSON

(From the Alfred I. du Pont Institute of The Nemours Foundation, Wilmington, Delaware)

(Received for publication, September 27, 1954)

Since the isolation of hydroxylysine as the picrate from the phosphotungstic acid-precipitable fraction of gelatin acid hydrolysates by Van Slyke, Hiller, Dillon, and MacFadyen (1), other isolation procedures have been reported (2, 3). The most recent is that of Sheehan and Bolhofer (4) who used chromatographic techniques in part to obtain analytically pure hydroxylysine from gelatin.[1] The present paper describes a chromatographic procedure whereby, without preliminary removal of cystine or arginine, all of the hydroxylysine in a hydrochloric acid hydrolysate of 100 gm. of gelatin was separated from the other amino acids. Separation was partially effected on a column of Amberlite IR-120 and was completed on a column of Dowex 50. From the effluent of the Dowex column, hydroxylysine monohydrochloride of 98 per cent purity was isolated in yields of 80 to 90 per cent. A single crystallization from aqueous alcohol gave analytically pure but partially racemized hydroxylysine in yields of 70 to 85 per cent of the original material.

The hydroxylysine from gelatin was resolved by chromatography into hydroxylysine and allohydroxylysine, a result in accord with Piez (6) who separated a synthetic mixture of hydroxy-D-lysine from allohydroxy-D-lysine similarly. Piez also made the observation that approximately 20 per cent of the hydroxylysine in acid hydrolysates of tooth collagen was allohydroxylysine. The present study indicates that allohydroxylysine is an

[1] Sheehan and Bolhofer (4) based their yields on the hydroxylysine, assayed by phosphotungstic acid precipitation, in the effluent fraction from a column of Amberlite IR-4B and obtained 65 to 75 per cent analytically pure hydroxylysine monohydrochloride. Computed on the basis of 1.2 gm. of free base per 100 gm. of protein reported by Rees (5) and which appears to be maximal, the yield of these authors is 48.4 per cent. Precipitation with phosphotungstic acid is a much less sensitive test for hydroxylysine than for lysine or arginine; hence considerable losses of hydroxylysine may occur undetected. Assay for hydroxylysine by the extremely sensitive periodate-chromotropic acid reaction (*vide infra*) probably accounts for the higher yields reported in the present paper. The authors are indebted to Dr. M. W. Rees, School of Biochemistry, University of Cambridge, Cambridge, England, for details and permission to use a recent unpublished method for the determination of hydroxylysine.

XUM

artifact arising through epimerization of hydroxylysine during the course of acid hydrolysis of the gelatin. In several experiments, after hydrolysis of the protein in 6 N hydrochloric acid for 16 hours, the amount of allohydroxylysine ranged from 7 to 20 per cent of the total, the amount of normal hydroxylysine being reduced correspondingly. On refluxing the hydrolysate for 96 hours, an equilibrium between the diastereoisomers appeared to be attained with 45 per cent hydroxylysine and 55 per cent allohydroxylysine.[2]

Isolation of Hydroxylysine from Gelatin Hydrolysates

Preparation of Acid Hydrolysates—100 gm. of gelatin (Nutritional Biochemicals Corporation) containing 8 per cent moisture were hydrolyzed under a reflux in 1500 ml. of 6 N hydrochloric acid for 16 hours. 5 gm. of carbon (Darco G-60, Darco Corporation, New York) were added and excess acid was removed in a rotating evaporator (8). The viscous residue was dissolved in water and the carbon was filtered and washed on the filter with 5 liters of water. The combined filtrate and washings, which were practically colorless, were made up to 10 liters. Prolonged evaporation of the hydrolysate gave maximal removal of free hydrochloric acid; thus the pH of the diluted solution was approximately 1.7 (glass electrode) and was suitable without further adjustment for introduction into the Amberlite column in the next step of the procedure.

Separation of Hydroxylysine Fraction on Amberlite IR-120 Column—The diluted hydrolysate was passed at a rate of 0.06 ml. per ml. of resin per minute through a 3.4 × 117 cm. (fully regenerated and back-washed) column of Amberlite IR-120[3] maintained at room temperature. All of the amino acids were adsorbed on the column and the effluent was discarded. The major portions of the neutral and acidic amino acids were eluted with 1 N hydrochloric acid (at the rate of 0.01 ml. per ml. of resin per minute) and discarded. After each 100 ml. of effluent had passed through, 0.5 ml. was collected, neutralized with NaOH, and treated with periodate at pH 7.5 according to Nicolet and Shinn (9) for the determination of α-amino-β-hydroxy acids. Formaldehyde evolved from serine or hydroxylysine was determined colorimetrically with chromotropic acid according to Rees (5).

[2] Reported to the American Society of Biological Chemists, Atlantic City, New Jersey, April, 1954 (7).

[3] Amberlite IR-120 (Rohm and Haas Company, Philadelphia, Pennsylvania) was conditioned by washing new resin with water until the effluent was colorless. It was converted to the hydrogen form by 50 m.eq. of hydrochloric acid per ml. of resin (10 liters of 4 N hydrochloric acid, passed at a rate of 0.133 ml. per ml. of resin per minute). The first regeneration sometimes necessitated a larger volume of acid to obtain a completely colorless effluent. The excess acid was washed out rapidly by 4 to 6 liters of water. A column of Amberlite IR-120 shrinks 15 to 18 per cent on acid regeneration but expands to its original volume with the final water wash.

This method, capable of detecting 0.01 μmole of formaldehyde (or amino acid equivalent) was employed to show that serine was first completely eluted by 1 N hydrochloric acid. After the passage of 9 to 10 liters, traces of hydroxylysine began to appear in the effluent. The developing fluid was then changed to 2.5 N hydrochloric acid and the effluent collected until a negative test for hydroxylysine was obtained. This was usually a little less than 3000 ml. This fraction contained all of the hydroxylysine and relatively small amounts of neutral amino acids, together with much lysine and some arginine, histidine, and ornithine.[4] It was evaporated to dryness.

Separation of Hydroxylysine on Dowex 50 Column—The dried residue was dissolved in 1 liter of water. This solution of pH 1.7 (glass electrode) was passed at a rate of 0.002 ml. per ml. per minute through a 7.5 × 30 cm. column of sodium Dowex 50[5] maintained at room temperature. The effluent was discarded. The column was then developed with 0.3 M acetate buffer, pH 5.0,[6] and the effluent was collected in 100 ml. fractions. The position of the emerging amino acids was determined by analysis of 0.1 ml. of each fraction by the colorimetric ninhydrin method (13). As described by Moore and Stein (11), monoamino acids emerged first. Since glucosamine and tryptophan are absent from gelatin, the next major peak was hydroxylysine, followed by ornithine and lysine (*cf.* (10)). Ammonia and arginine were left on the column and discarded later in the alkali-regenerating fluid used preparatory to another run. The position of hydroxylysine was confirmed by the Rees procedure and that of ornithine by the method of Chinard (14). Fig. 1 shows a typical chromatogram.

As shown in Fig. 1, the separation of hydroxylysine was good and, provided that elution was kept to the recommended flow rate or the column was not overloaded, overlapping of amino acids did not occur.[7] In other runs,

[4] Ornithine was found by Hamilton and Anderson (10) in acid hydrolysates of some gelatins. It was considered an artifact which arises from alkaline degradation of arginine during the manufacturing process and does not seem to be a normal constituent of collagenous tissue.

[5] The sodium Dowex 50 (8 per cent cross-linked, 200 to 400 mesh, The Dow Chemical Company, Midland, Michigan) was prepared according to Moore and Stein (11) and equilibrated with 0.2 M acetate buffer, pH 3.4, prior to introduction of the amino acid solution.

[6] Stock 0.4 M acetate solution, pH 5.0, was made by dissolving 64 gm. of solid NaOH and 135 ml. of glacial acetic acid in water and making up to 4 liters. To prepare 0.2 M solution, pH 3.4, 357 ml. of 1 N hydrochloric acid were added to 1 liter of 0.4 M solution and made up to 2 liters. The 0.3 M buffer was made from the stock solution by suitable dilution with water. As recommended by Hirs, Moore, and Stein (12), detergent and sequestering agent were omitted from these buffers.

[7] The capacity and resolving power of both the Amberlite IR-120 and Dowex 50 columns described are adequate for treatment of 100 to 300 gm. of hydrolyzed gelatin; larger quantities overload the Dowex 50 column especially and the resolution of hydroxylysine is inadequate.

some tailing of the hydroxylysine occurred, but, as these later fractions contained only a few mg. of amino acid, they were discarded. The hydroxylysine-containing fractions were combined.

Isolation of Hydroxylysine Monohydrochloride—The combined fractions were evaporated to 100 ml. and an equal volume of concentrated hydrochloric acid was added and again evaporated. Another 100 ml. of concentrated hydrochloric acid were added to the residue and the bulky precipitate of sodium chloride was filtered and washed with three 100 ml.

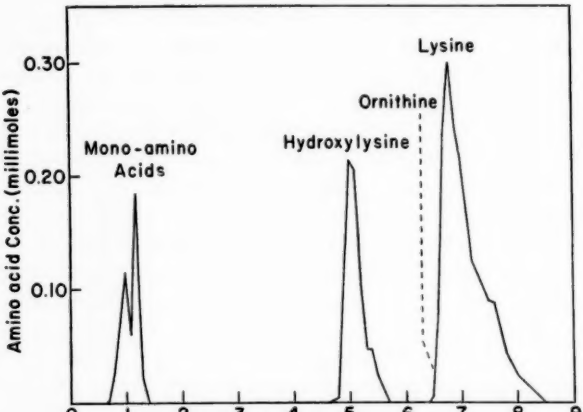


FIG. 1. Separation of hydroxylysine on a column of Dowex 50, 7.5 × 30 cm., operated in the sodium form at 25°, with 0.3 M acetate buffer, pH 5.0, as eluent. The amino acid mixture placed on the column is the hydroxylysine-containing effluent fraction from a column of Amberlite IR-120 employed to give a preliminary partial separation of hydroxylysine from other amino acids in the gelatin hydrolysate.

portions of concentrated hydrochloric acid; the combined filtrate and washings were evaporated to dryness. The residue was stirred with 100 ml. of ethyl alcohol in which the hydroxylysine dihydrochloride dissolved slowly. Undissolved sodium chloride was removed by filtration and washed with a few ml. of alcohol and the filtrate and washings were evaporated to dryness. The residue was dissolved in 20 ml. of water and the solution neutralized with lithium carbonate to pH 6.5.[8] This solution was evaporated to a viscous syrup. Methyl alcohol (50 ml.) was added to the

[8] Conversion of the dihydrochloride to the monohydrochloride in aqueous solution by lithium carbonate was found preferable to conversion in alcoholic solution with pyridine or aniline. The latter procedures gave very gelatinous precipitates which stood for 24 hours in solution before granulating, and the yields were lower.

syrup and agitated with glass beads to loosen material adhering to the flask wall. The lithium chloride and some of the hydroxylysine dissolved in the methyl alcohol; the remainder of the hydroxylysine was suspended. 8 volumes of acetone added to the solution precipitated the dissolved hydroxylysine. After standing for 1 hour, the hydroxylysine was collected on a filter and washed with abundant acetone to remove residual lithium chloride. It was finally washed with 50 ml. of ethyl alcohol[9] and dried *in vacuo* over calcium chloride.

In typical experiments, the yields of hydroxylysine monohydrochloride ranged from 80 to 90 per cent based on the highest value reported in the literature (5) of 1.2 gm. of free base per 100 gm. of protein (1.35 gm. of hydroxylysine monohydrochloride per 100 gm. of gelatin containing 8 per cent moisture). The product melted with decomposition at approximately 210° and the specific rotation ranged from $[\alpha]_D^{25}$ +1.19° to +12.1° (c = 2 per cent, 6 N hydrochloric acid) for the free base. The product was uncontaminated with other amino acids, as shown by chromatographic analysis on a 15 cm. column of Dowex 50 (11). Elementary analysis gave results within 2 to 3 per cent of theoretical. To recrystallize, the material was dissolved in 10 ml. of water, decolorized with carbon, and filtered, and ethyl alcohol was added until the cloud point was reached (approximately 60 per cent alcohol). It was seeded and stored at 4° for 12 hours. The alcohol concentration was then increased to 70 per cent. Two more additions of alcohol at 12 hour intervals brought the concentration to 90 per cent (*cf.* Sheehan and Bolhofer (4)). After the last addition of alcohol, crystallization was allowed to continue a further 24 hours at 4°. The crystals were collected, washed with a little 90 per cent alcohol and absolute alcohol, and then dried *in vacuo* over calcium chloride. This method of recrystallization gave recoveries of 85 to 90 per cent of the crude material or 70 to 85 per cent of the hydroxylysine in the 100 gm. of gelatin. The melting point was 221–225° (literature 225° (4, 15, 16)) and the specific rotation ranged from $[\alpha]_D^{25}$ +6.87° to +14.1° (c = 2 per cent, 6 N hydrochloric acid) for the free base. Hydroxylysine with a specific rotation of $[\alpha]_D^{25}$ +17.8° reported by Fones (17) for the pure L enantiomorph was never obtained for reasons detailed below.

$C_6H_{12}O_3N_2Cl$.	Calculated.	C 36.27, H 7.61, N 14.10, Cl 17.85
	Found.	" 36.30, " 7.73, " 14.20, " 17.98

Chromatographic Resolution of Hydroxylysine Diastereoisomers—250 mg.

[9] The solubility of hydroxylysine monohydrochloride in absolute methyl alcohol is approximately 0.2 mg. per ml. at room temperature, and in ethyl alcohol 0.006 mg. per ml.; in acetone it is practically insoluble. Methyl alcohol was accordingly avoided for washing or drying precipitates.

of hydroxylysine monohydrochloride (from 16 hour gelatin hydrolysate), $[\alpha]_D^{25}$ +10.9° (free base), were dissolved in 25 ml. of water and adjusted to pH 2 with hydrochloric acid. The solution was washed onto a 3.4 × 115 cm. column of sodium Dowex 50, buffered at pH 3.4, and the column was developed at room temperature with 0.1 M sodium phosphate buffer, pH 7.5.[10] The first 4.5 liters of effluent were discarded and the next 2.0 liters were collected in 10 ml. fractions.

The position and shape of the resulting peaks were established by ninhydrin colorimetric analysis of 0.1 ml. samples taken from alternate fractions. The resolution obtained was similar to that shown at *A* (Fig. 2), with 91 per cent of the recovered hydroxylysine in the fore peak and 9 per cent in the after peak.[11] (Recovery of hydroxylysine from the column in this experiment was 96 per cent.) Fractions comprising the fore and after peaks, respectively, were combined; fractions that overlapped were discarded. The combined fore and after peak fraction solutions, each approximately 300 ml., were passed through 2.2 × 30 cm. columns of Amberlite IR-120 in the hydrogen form, conditioned as previously described. Hydroxylysine and sodium were retained on the column, while some detergent (BRIJ 35) and phosphoric acid appeared in the effluent. The remainder of both these latter compounds was washed out with 2000 ml. of water. All but a trace of sodium was preferentially eluted as sodium chloride with 450 ml. of 1 N hydrochloric acid: the hydroxylysine was retained on the column as described by Stein (18). The hydroxylysine was then eluted with 200 ml. of 4 N hydrochloric acid and the effluent evaporated to dryness. Hydroxylysine monohydrochloride was isolated and crystallized as previously described. The specific rotation of the fore peak material was $[\alpha]_D^{25}$ +16.5° (c = 2.0 per cent, 6 N hydrochloric acid) for the free base and was unchanged after recrystallization. The specific rotation of the after peak material was $[\alpha]_D^{25}$ −27.1° (c = 2.31 per cent, 6 N hydrochloric acid) for the free base. Fones (17) reported $[\alpha]_D^{25}$ +17.8° for the hydroxy-L-lysine free base and $[\alpha]_D^{25}$ −32.1° for the allohydroxy-D-lysine free base. The values obtained in the present work indicate that the larger fore peak was hydroxy-L-lysine and the smaller after peak was allohydroxy-D-lysine. The relative positions assigned to the normal and allo forms are in agreement with those of hydroxylysine and allohydroxylysine observed by Piez (6).

A commercial synthetic racemic hydroxylysine was resolved similarly into 40 per cent hydroxy-DL-lysine and 60 per cent allohydroxy-DL-lysine;

[10] As a detergent, 10 ml. of BRIJ 35 (Atlas Powder Company, Wilmington, Delaware) solution (11) were added to each 1000 ml. of buffer.

[11] In other similar experiments the allohydroxylysine in 16 hour hydrolysates ranged from 7 to 20 per cent of the total hydroxylysine. In 25 hour hydrolysates the values were more consistent and agreed closely with the results found after hydrolysis of hydroxy-DL-lysine for the same length of time.

approximately 20 per cent only of this preparation was therefore hydroxy-L-lysine.

Inversion of Hydroxylysine and Allohydroxylysine—Commercial racemic hydroxylysine was resolved chromatographically into hydroxy-DL-lysine

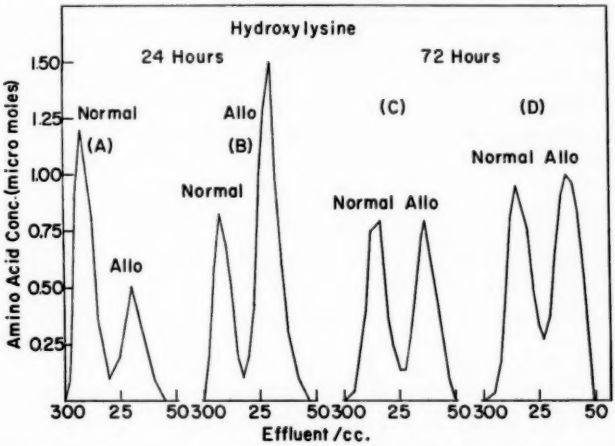


FIG. 2. Chromatograms showing inversion of hydroxylysine (*A* and *C*) and allohydroxylysine (*B* and *D*) refluxed for 24 and 72 hours in 6 N hydrochloric acid. For experimental details see the text. *A*, hydroxylysine 68 per cent-allohydroxylysine 32 per cent; *B*, hydroxylysine 32 per cent-allohydroxylysine 68 per cent; *C*, hydroxylysine 53 per cent-allohydroxylysine 47 per cent; *D*, hydroxylysine 45 per cent-allohydroxylysine 55 per cent.

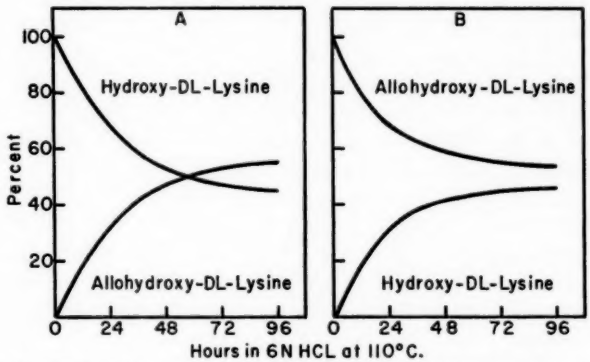


FIG. 3. Inversion of hydroxy-DL-lysine (*A*) and allohydroxy-DL-lysine (*B*) on refluxing in 6 N hydrochloric acid. The ordinates give the percentage of each diastereoisomer present at the times indicated (abscissas).

and allohydroxy-DL-lysine and each diastereoisomer isolated as described above. Both were chromatographically homogeneous. Respective portions were refluxed for 24, 48, 72, and 96 hours in 6 N hydrochloric acid and then rechromatographed. Chromatograms for the portions refluxed for 24 and 72 hours are shown in Fig. 2. The results are summarized in Fig. 3.

It is seen that commencing with either hydroxylysine or allohydroxylysine after 96 hours an equilibrium appeared to be attained with 46 per cent hydroxylysine-54 per cent allohydroxylysine. After refluxing in 1 N barium hydroxide for 24 or 48 hours, the relative proportions were 44 per cent hydroxylysine-56 per cent allohydroxylysine.

DISCUSSION

The evidence presented indicates that optical inversion of hydroxylysine occurs readily in boiling 6 N hydrochloric acid. It was also found that there was no marked difference between hydroxy-L-lysine (from gelatin) and hydroxy-DL-lysine (synthetic) with respect to rate of inversion and equilibrium attained. For example, a sample of hydroxy-L-lysine kindly supplied by Dr. John C. Sheehan, Massachusetts Institute of Technology, isolated from gelatin that had been hydrolyzed for 25 hours, was resolved into 35 per cent allo-65 per cent normal hydroxylysine. After being refluxed for a further 71 hours (96 hours total) in 6 N hydrochloric acid, it was resolved into 56 per cent allo-44 per cent normal hydroxylysine. Similarly, a calf skin gelatin hydrolyzed for 96 hours was chromatographed and resolved into 55 per cent allo-45 per cent normal hydroxylysine (see synthetic hydroxy-DL-lysine in Fig. 3, *A*). It seems justifiable to conclude that, since allohydroxylysine in gelatin hydrolysates approximates the amount found after refluxing hydroxylysine the same length of time in 6 N hydrochloric acid, the former is an artifact which arises during the course of acid hydrolysis of the protein. To obtain maximal values, resolution without isolation would be preferable because of the greater solubility of allohydroxylysine in water and alcohol and because of the partial separation of the diastereoisomers which occurs on crystallization from water-ethanol mixtures. For example, crude hydroxylysine, $[\alpha]_D^{25}$ +1.16° (free base), was crystallized in successive crops from the same solution as the alcohol concentration was raised from 60 to 70, 80, and 90 per cent; specific rotations were +7.06°, +3.50°, −8.04°, and −9.56°, respectively. The presence of 20 per cent of allohydroxylysine in tooth collagen hydrolysates as reported by Piez (6) seems to be adequately explained by the present findings.

Concerning the site of optical inversion, the data are consistent with the view that epimerization at the α-carbon atom predominates. The change from hydroxy-L-lysine to allohydroxy-D-lysine is compatible with the

specific optical rotations observed, +16.5° and −27.1°, respectively, for material isolated (after separation of the diastereoisomers) from the same source. Insufficient material was available to undertake resolution of the diastereoisomers into their optical antipodes to prove conclusively that racemization (*i.e.* epimerization at the δ-carbon atom) had or had not occurred. That both of the rotations given above are lower than those reported by Fones (17) for the pure substances indicates that some epimerization at the δ-carbon atom might have taken place. Without doubt, however, the major site of inversion would appear to be the α-carbon atom. This is in agreement with the evidence in the literature concerning racemization of amino acids containing two asymmetric centers (19).

The authors gratefully acknowledge much help and profitable discussion during the course of this work with Dr. S. Moore and Dr. W. H. Stein of The Rockefeller Institute for Medical Research, New York.

SUMMARY

1. A method is described whereby hydroxylysine was first partially separated from the bulk of the amino acids in a hydrochloric acid hydrolysate of gelatin on a column of Amberlite IR-120 cationic exchange resin. Resolution was completed on a column of sodium Dowex 50 with 0.3 M acetate, pH 5.0, as eluent. Hydroxylysine monohydrochloride (98 per cent analytically pure) was isolated in yields which ranged from 80 to 90 per cent of that originally present in gelatin. A single crystallization from water-ethanol gave an analytically pure product in yields of 70 to 85 per cent of the original material.

2. The diastereoisomers of hydroxylysine were resolved on 100 cm. columns of sodium Dowex 50 with 0.1 M phosphate, pH 7.5, as eluent.

3. Inversion of hydroxylysine and allohydroxylysine was shown to take place rapidly in boiling 6 N hydrochloric acid. After 96 hours, an apparent equilibrium of the diastereoisomers was attained with 46 per cent hydroxylysine-54 per cent allohydroxylysine. Hydroxy-L-lysine (from gelatin) and hydroxy-DL-lysine (synthetic) are inverted at approximately the same rate and attain similar equilibrium values.

4. Hydroxylysine from gelatin was resolved into hydroxy-L-lysine and allohydroxy-D-lysine. Inversion took place by epimerization predominantly at the α-carbon atom. It was not proved conclusively that epimerization at the δ-carbon atom did not occur.

BIBLIOGRAPHY

1. Van Slyke, D. D., Hiller, A., Dillon, R. T., and MacFadyen, D. A., *Proc. Soc. Exp. Biol. and Med.*, **38,** 548 (1938).
2. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35,** 307 (1941).

3. Heathcote, J. G., *Biochem. J.*, **42,** 305 (1948).
4. Sheehan, J. C., and Bolhofer, W. A., *J. Am. Chem. Soc.*, **72,** 2466 (1950).
5. Rees, M. W., *Biochem. J.*, **40,** 632 (1946).
6. Piez, K. A., *J. Biol. Chem.*, **207,** 77 (1954).
7. Hamilton, P. B., and Anderson, R. A., *Federation Proc.*, **13,** 224 (1954).
8. Craig, L. C., Gregory, J. D., and Hausmann, W., *Anal. Chem.*, **22,** 1462 (1950).
9. Nicolet, B. H., and Shinn, L. A., *J. Am. Chem. Soc.*, **61,** 1615 (1939).
10. Hamilton, P. B., and Anderson, R. A., *J. Biol. Chem.*, **211,** 95 (1954).
11. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192,** 663 (1951).
12. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **195,** 669 (1952).
13. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176,** 367 (1948).
14. Chinard, F. P., *J. Biol. Chem.*, **199,** 91 (1952).
15. Weisiger, J. R., *J. Biol. Chem.*, **186,** 591 (1950).
16. Van Slyke, D. D., Hiller, A., and MacFadyen, D. A., *J. Biol. Chem.*, **141,** 681 (1941).
17. Fones, W. S., *J. Am. Chem. Soc.*, **75,** 4865 (1953).
18. Stein, W. H., *J. Biol. Chem.*, **201,** 45 (1953).
19. Neuberger, A., *Advances in Protein Chem.*, **4,** 340 (1948).

PREPARATION AND ENZYMATIC HYDROLYSIS OF POLY-γ-ETHYLGLUTAMYL BOVINE PLASMA ALBUMIN*

By MAURICE GREEN† and MARK A. STAHMANN

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wisconsin)

(Received for publication, May 17, 1954)

One of the most interesting methods for modifying proteins, which has only recently been discovered, is the addition through peptide bond formation of amino acids and peptides to native proteins by reaction with *N*-carboxyamino acid anhydrides. This was introduced by Stahmann and Becker (1, 2) who allowed buffered aqueous solutions of bovine plasma albumin and chymotrypsin to react with *N*-carboxyglycine anhydride, whereby the molecular weights of both proteins were increased by about 12 per cent. It was surprising that, despite the large increase in glycine content, there was no appreciable change in several of the physiological properties of the modified proteins; the chymotrypsin derivative still retained enzymatic activity, and the polyglycyl albumin was precipitated by an antiserum prepared against normal plasma albumin.

Since the properties of proteins are known to depend to a large extent upon polar groups in the molecule provided by acidic and basic amino acids, it was of interest to study the addition of glutamic acid peptides to a protein. In the work reported here, α-bonded glutamic acid peptides containing esterified γ-carboxyl groups were attached to bovine plasma albumin by means of peptide bonds formed upon reaction of the protein with the *N*-carboxy anhydride of γ-ethyl glutamate, and some of the physical and chemical properties of the resulting modified protein were studied. The susceptibility to the action of several proteolytic enzymes of this modified protein, as well as polyglycyl albumin, was also investigated.

EXPERIMENTAL

Preparation of Poly-γ-ethyl-L-glutamyl Bovine Plasma Albumin—5 gm. of crystalline bovine plasma albumin[1] (BPA) were dissolved in 500 ml. of

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a research grant (No. E-101) from the National Microbiological Institute of the National Institutes of Health, United States Public Health Service.

† Present address, Research Department, the Children's Hospital of Philadelphia, Philadelphia, Pennsylvania.

[1] Purchased from Armour and Company, Chicago, Illinois.

M/15 phosphate buffer, pH 7.3, and cooled to 3°. 5 gm. of *N*-carboxy-γ-ethyl-L-glutamate anhydride (3) were added with stirring, the solution becoming turbid and carbon dioxide being released. The reaction mixture was shaken for 4 hours at room temperature and kept at 3° for 20 additional hours. The turbid reaction mixture was centrifuged at 17,000 × *g* for 30 minutes to remove insoluble polymerization products and then dialyzed with stirring against several changes of distilled water at 3° for 4 days to remove γ-ethyl glutamate and soluble peptides. The poly-γ-ethylglutamyl BPA was recovered from the dialysate by lyophilization to yield 5.0 gm. of white, fluffy solid. Control experiments were made in which no anhydride was added and also in which the anhydride was polymerized in buffer and then mixed with BPA.

Glutamic Acid Analysis—The amount of γ-ethyl glutamate added to BPA was determined by analysis of the protein hydrolysate for the increased glutamic acid content with a glutamic acid decarboxylase preparation. The CO_2 released from the glutamic acid was measured in a Warburg respirometer. The enzyme was prepared from *Escherichia coli*, NCTC strain 4157, as described by Umbreit and Gunsalus (4). All analyses were computed on a dry weight basis (constant weight at 110°) and a molecular weight of BPA of 69,000. Appropriate corrections were applied for low recovery from glutamic acid controls (86 to 89 per cent) and higher than theoretical amounts of CO_2 evolved from BPA hydrolysates (114 to 119 per cent).

Alkaline Saponification of Poly-γ-ethylglutamyl Albumin—Poly-γ-ethylglutamyl BPA was treated with dilute alkali under nitrogen for different periods of time. All reagents employed were CO_2-free. The number of ester groups saponified as revealed by alkali consumption was determined by back-titrating the alkaline reaction mixture of poly-γ-ethylglutamyl BPA under nitrogen with HCl to pH 6.0 by using an ultramicro burette and a Beckman model G pH meter equipped with a glass electrode from pH 0 to 11. The extent of ester hydrolysis was calculated from the difference between the amounts of acid required to back-titrate poly-γ-ethylglutamyl BPA and native BPA.

Electrophoretic Analysis[2]—This was performed on the Spinco model H electrophoresis-diffusion apparatus equipped with a Tiselius cell of 11 ml. capacity in a bath at 1°.

Enzymatic Hydrolysis of Modified Proteins—Appropriate buffered volumes of freshly prepared solutions of protein and enzyme were mixed and incubated at 37°. Suitable aliquots were removed at zero time and at convenient intervals and analyzed to determine the extent of hydrolysis. Control experiments were included with no substrate and with no enzyme.

[2] Appreciation is extended to Dr. R. M. Bock for generous advice on the electrophoretic analysis.

Papain and pancreas extract solutions were prepared as previously described (3). Crystalline trypsin,[1] chymotrypsin,[1] and pepsin[1] were dissolved in buffer for use. The crude peptidase preparation consisted of a glycerol extract of hog intestinal mucosa prepared according to Johnson *et al.* (5).

The polyglycyl BPA used in the enzyme studies was kindly supplied by Dr. R. R. Becker. The molecular weight of this preparation was calculated to be 81,000 from microbiological assay for the glycine content of the protein and a molecular weight of 69,000 for native BPA.

Triglycine was kindly provided by Dr. M. J. Johnson. The ninhydrin color yield of the tripeptide on a molar basis relative to leucine was found to be 0.92. The color yields of diglycine and glycine have been reported as 0.89 and 1.01, respectively (6).

The ninhydrin color procedure (6) for amino nitrogen was used in following the hydrolysis of the modified proteins by trypsin, chymotrypsin, pepsin, and the peptidase preparation. This method has the advantage of great sensitivity which is useful in following hydrolysis in small samples. Although the ninhydrin color yields given by free amino acids may not be the same as when these amino acids are situated at the amino end of peptide chains, such as in an enzymatic hydrolysate, a useful comparison can be made of the colors given by the enzymatic hydrolysates obtained by the action of an enzyme on the native and modified proteins. In several cases (7, 8), results obtained by the ninhydrin color procedure on proteins or protein hydrolysates were shown to be in agreement with the results of α-amino nitrogen analysis. α-Amino nitrogen analysis was used here in several experiments to verify further the results obtained with the ninhydrin procedure. Good agreement was found between the two methods for the per cent reduction in the hydrolysis of poly-γ-ethylglutamyl BPA as compared to unmodified BPA by trypsin and chymotrypsin.

Triplicate 0.100 ml. aliquots of buffer, standard leucine, native BPA incubation mixture, and modified BPA incubation mixtures were pipetted into a series of photometer tubes containing 1 ml. of ninhydrin reagent, the color was developed as described by Moore and Stein (6), and readings were taken on the Beckman model B spectrophotometer at 570 mμ. The results were expressed in terms of the equivalents of leucine standard corresponding to the given color value. A straight line relationship was found between optical density and standard leucine concentration under the condition employed up to 12.2 mmoles of leucine.

Results

Synthesis—Poly-γ-ethyl-L-glutamyl bovine plasma albumin was prepared by treatment of BPA with an equal weight of γ-ethyl-L-glutamate anhydride in phosphate buffer at pH 7.3. As calculated from the amount of

γ-ethyl glutamate added to BPA (Table I), the glutamic acid content of the protein was increased by 110 per cent, and the molecular weight of the protein increased by 19.5 per cent. The glutamic acid content of the dialyzed poly-γ-ethylglutamyl BPA was not decreased by exhaustive electrodialysis, thus providing evidence that the amino acid was chemically attached and not just reversibly bound to the protein.

Treatment with Alkali—Preliminary experiments were made treating poly-γ-ethylglutamyl BPA and native BPA with dilute alkali for different periods of time in order to investigate conditions for the hydrolysis of the γ-ethyl ester groups to free carboxyl groups. The number of free carboxyl groups formed and the solubility of the resulting protein derivative between pH 2 and 11 are summarized in Table II. It is seen that treatment with

TABLE I

Addition of γ-Ethyl Glutamate to BPA

Protein	γ-Ethyl glutamate added per 100 gm. protein	Mol. wt.	Glutamic acid per mole protein	Added glutamic acid per mole protein
	gm.		*moles*	*moles*
BPA		69,000	77*	
Poly-γ-ethylglutamyl BPA	19.5	82,500†	163	86
" " ‡	19.5	82,500†	163	86

* Calculated from data of Stein and Moore (9).

† Calculated from the increase in γ-ethyl glutamate content.

‡ This preparation, in addition to being dialyzed, was exhaustively electrodialyzed.

0.05 N NaOH resulted in a maximum of 80 per cent hydrolysis of the ester groups in 1 to 2 hours.

Native BPA and poly-γ-ethylglutamyl BPA were completely soluble from pH 2 to 11 (Table II) under the conditions tested. Treatment of native BPA with 0.05 N NaOH for 1 or 2 hours did not convert it to an insoluble protein. However, poly-γ-ethylglutamyl BPA, upon treatment with 0.05 N NaOH for as little as 30 minutes, became insoluble in the pH region 5 to 2.5, which encompasses the estimated isoelectric region of the new modified protein (10). It cannot be said whether this decreased solubility is due to a change in the parent structure caused by the alkali or to the large increase in the number of charged γ-carboxyl groups on the protein molecule. In this respect, it is pointed out that chemical modification of proteins by reaction with organic compounds under mild conditions may produce changes in the physical properties of the protein without denaturation (11).

Electrophoretic Analysis—Electrophoresis studies were made on poly-γ-ethylglutamyl BPA and its alkali-treated product. The electrophoretic patterns of the protein derivatives are shown in Fig. 1 and the corresponding calculated mobilities are given in Table III. Poly-γ-ethylglutamyl BPA showed a single peak with a 9 per cent increase in mobility over BPA in Veronal buffer at pH 8.6. An increase in mobility would be expected,

TABLE II

Treatment of BPA and Poly-γ-ethylglutamyl with Alkali

Protein	Treatment*	No. free carboxyl groups liberated per molecule	Per cent ester hydrolysis	Solubility† after treatment, pH range 2–11
BPA	No treatment			Soluble
"	0.05 N NaOH, 1 hr.			"
"	0.05 " " 2 hrs.			"
"	0.1 " " 19 "‡			Insoluble, pH 5.0–3.3
Poly-γ-ethylglutamyl BPA	No treatment			Soluble
" "	0.05 N NaOH, 30 min.	37	45	Insoluble, pH 4.5–3.0
" "	0.05 " " 1 hr.	50	60	Insoluble, pH 4.6–2.7
" "	0.05 " " 2 hrs.	67	80	Insoluble, pH 4.7–2.7
" "	0.05 " " 4 "	66	80	Insoluble, pH 4.7–2.5
" "	0.1 " " 22 "§	81	95	Insoluble, pH 4.9–2.0

* Room temperature (25–28°) unless otherwise specified.

† Protein concentration approximately 0.6 per cent in about 0.15 M NaCl solution.

‡ Treatment at 3°.

§ Treatment at 3° for 19 hours and then at 23° for 3 hours.

since, in the formation of poly-γ-ethylglutamyl BPA, ε-amino groups of lysine (pK = 9.4 (13)) in BPA are acylated by reaction with *N*-carboxy γ-ethyl glutamate anhydride and replaced by the terminal α-amino groups (pK = 8.0 (13)) of the added glutamic acid peptides. The result of this exchange of ε-amino for α-amino groups is a decrease in positive charge and thus an increase in mobility of the negatively charged protein at pH 8.6.

The electrophoretic pattern of alkali-treated poly-γ-ethylglutamyl BPA showed a slight shoulder and revealed an increase in mobility of 1.8×10^{-5}

mobility unit with respect to untreated poly-γ-ethylglutamyl BPA (a 25 per cent increase in mobility). Since this preparation contained 60 newly formed completely ionized γ-carboxyl groups (at pH 8.6), it was of interest

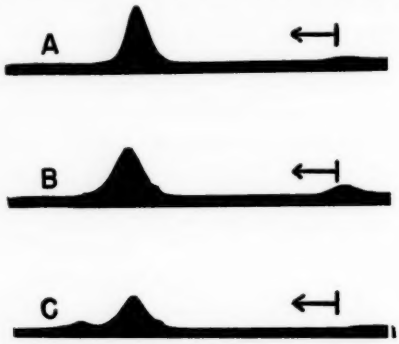


FIG. 1. Electrophoretic patterns in descending limb at pH 8.6 in Veronal buffer, 0.1 ionic strength. *A*, poly-γ-ethylglutamyl BPA at 131 minutes. *B*, poly-γ-ethylglutamyl BPA (treated with 0.05 N NaOH for 1 hour) at 119 minutes. *C*, BPA (treated with 0.05 N NaOH for 1 hour) at 199 minutes.

TABLE III

Mobilities of BPA Derivatives at pH 8.6 in Veronal Buffer, 0.1 Ionic Strength

Preparation	Mobility × 10^{-5} cm.^{2} per volt per sec. in descending limb	Change in mobility*	Remarks
		per cent	
BPA	−6.50†		Single peak
Poly-γ-ethylglutamyl BPA (Fig. 1, *A*)	−7.07	+9	" "
" " 1 hr. in 0.05 N NaOH‡ (Fig. 1, *B*)	−8.83	+36	Slight shoulder
BPA, 1 hr. in 0.05 N NaOH (Fig. 1, *C*)	−5.40	−17	Main component
	−6.70	+3	10% of total

* Compared with unmodified BPA.

† From data of Alberty (12).

‡ Contained 60 new γ-carboxyl groups.

to calculate the increase in mobility that would be expected from such a large increase in negative charge. On employing the value of 0.2×10^{-5} cm.^{2} per volt per second per charge for bovine plasma albumin (10), one would expect from 60 added carboxyl groups an increase in mobility of $60 \times 0.2 \times 10^{-5} = 12 \times 10^{-5}$ cm.^{2} per volt per second. An increase of only 1.8×10^{-5} was observed, which is only 15 per cent of that calculated above.

Native BPA, after treatment with alkali, showed two components upon electrophoretic analysis. The main component had a 17 per cent decrease in mobility and may represent a soluble, alkali-denatured form of the protein. The small amount of faster moving component (about 10 per cent) had about the same mobility as native BPA and may be a small residue of undenatured BPA.

Enzymatic Hydrolysis of Modified Proteins—The modified proteins used in a study of their susceptibility to the action of several proteolytic enzymes were poly-γ-ethylglutamyl BPA of molecular weight 82,500 and polyglycyl BPA of molecular weight 81,000. The proteolytic enzyme preparations employed include papain, pancreas extract, a crude peptidase preparation, and crystalline trypsin, chymotrypsin, and pepsin.

TABLE IV

Hydrolysis of Poly-γ-ethylglutamyl and Polyglycyl BPA by Papain and Pancreas Extract

Incubation mixtures were made up in 0.2 M acetate buffer, pH 5.0, and contained 9 mg. of protein substrate per ml. and 10 per cent of the enzyme extract. Papain mixtures contained 9 mg. of sodium thioglycolate activator per ml. The extent of hydrolysis was determined by α-amino nitrogen analysis on 2.0 ml. aliquots of the reaction mixture.

Substrate	Increase in α-amino N, μmoles α-NH_2-N per mg. substrate	Per cent reduction in hydrolysis
Papain, 26 hrs.		
BPA	4.37	
Poly-γ-ethylglutamyl BPA	3.31	25
Polyglycyl BPA	3.43	20
Pancreas extract, 39 hrs.		
BPA	3.41	
Poly-γ-ethylglutamyl BPA	2.55	25
Polyglycyl BPA	2.71	20

Table IV illustrates the hydrolysis of modified proteins by papain and pancreas extract. With both enzyme preparations, there is a decrease in the extent of hydrolysis of 20 to 25 per cent for both modified proteins as compared to native BPA. Since these enzyme preparations contain various proteolytic enzymes of different specificities, it is likely that the glutamic acid and glycine residues attached to the protein were hydrolyzed to some extent. In this respect, it is pointed out that polyglutamic acid is hydrolyzed by both pancreas extract and papain (3).

TABLE V

Hydrolysis of Poly-γ-ethylglutamyl and Polyglycyl BPA by Crystalline Trypsin, Chymotrypsin, and Pepsin

Trypsin and chymotrypsin incubation mixtures were prepared in M/15 phosphate buffer, pH 7.3, and contained 8 mg. of protein substrate per ml. Trypsin reaction mixtures had 0.05 mg. of enzyme per ml. and chymotrypsin mixtures 0.03 mg. per ml. Pepsin reaction mixtures consisted of 8 mg. of protein substrate and 0.18 mg. of pepsin per ml. of 0.2 M phosphate buffer, pH 2.0. Hydrolysis determined by photometric ninhydrin analysis.

Substrate	Increase in ninhydrin color,* μmoles per mg. substrate	Per cent reduction in hydrolysis
Trypsin, 17 hrs.		
BPA	0.429	
Poly-γ-ethylglutamyl BPA	0.170	60
Polyglycyl BPA	0.172	60
Chymotrypsin, 23 hrs.		
BPA	0.745	
Poly-γ-ethylglutamyl BPA	0.226	70
Polyglycyl BPA	0.351	55
Pepsin, 24 hrs.		
BPA	1.03	
Poly-γ-ethylglutamyl BPA	1.03	0
Polyglycyl BPA	1.02	0

* Ninhydrin color expressed in terms of micromoles of leucine standard.

TABLE VI

Effect of Crude Peptidase Preparation on Poly-γ-ethylglutamyl and Polyglycyl BPA

Incubation mixtures contained 0.3 mg. of triglycine per ml. or 4 mg. of protein substrate per ml. and 20 per cent peptidase preparation in M/15 phosphate buffer, pH 8.0. Hydrolysis determined by photometric ninhydrin analysis.

Substrate	Time	Per cent hydrolysis
	hrs.	
Triglycine	1	140*
"	16	200*
BPA	24	0
Poly-γ-ethylglutamyl BPA	24	0
Polyglycyl BPA	24	0

* Complete hydrolysis of the two peptide bonds is 200 per cent.

Table V presents data obtained on the hydrolysis of modified proteins by the crystalline enzymes trypsin, chymotrypsin, and pepsin. With trypsin and chymotrypsin there is a decrease of 55 to 70 per cent in the extent of hydrolysis of both modified proteins as compared to unmodified BPA. However, with pepsin, there appears to be the same degree of hydrolysis of modified albumins and native BPA.

Table VI summarizes the results of experiments on the susceptibility of the modified albumins to the action of a crude peptidase preparation obtained from hog intestinal mucosa. Triglycine was hydrolyzed completely to free glycine, indicating that the enzyme preparation had high tripeptidase and dipeptidase activity for glycine peptides. However, no hydrolysis was observed on polyglycyl BPA, poly-γ-ethylglutamyl BPA, or unmodified BPA. The lack of hydrolysis of polyglycyl BPA discloses that glycine peptides when attached to BPA are not split by this active peptidase preparation.

DISCUSSION

Becker and Stahmann (2) demonstrated that, in their preparation of polyglycyl albumin which contained 171 moles of added glycine per mole of protein, one-third of the free amino groups of albumin had been acylated. This indicated that the glycine was attached in the form of peptides having an average length of 7 residues, assuming that only the amino groups of the protein had reacted. The preparation of polyglycyl albumin employed in the enzymatic studies was prepared under similar conditions except that a large ratio of anhydride to protein was used, thus resulting in more glycine being added to the albumin. The reaction of bovine plasma albumin with an equal weight of *N*-carboxy γ-ethyl glutamate resulted in the addition of 86 moles of γ-ethyl glutamate per mole of albumin. If it is assumed that only amino groups of the protein have reacted and that one-third of these has been acylated, it can then be calculated that the glutamic acid is attached on the average as tetrapeptides. Although there is sufficient glutamic acid added (86 moles per mole of protein) to react with all the free amino groups of the protein, it is unlikely that each amino group is acylated because of the great reactivity of the anhydride molecule. It is more probable that the more sterically available amino groups are first acylated, the added γ-ethyl glutamate residues being subsequently further acylated in a stepwise manner by anhydride resulting in an elongation of the peptide chain. The albumin molecule is thus modified by the attachment of a number of peptide chains at various loci.

Although it would not be anticipated that protein denaturation would occur during the mild conditions employed to prepare poly-γ-ethylglutamyl BPA, some of the physical properties of this derivative were ex-

amined to test this possibility briefly. The processes involved in denaturation are complex and a precise definition of the term is difficult. In the case of chemically modified proteins, it is often difficult to ascertain whether observed changes in physical properties are caused by the chemical modification or by alterations in protein structure produced by denaturation. The lack of denaturation of a protein can only be demonstrated in a negative sense, that is a protein can be demonstrated to be denatured but cannot be conclusively shown to be undenatured. Three simple physical criteria were employed to examine poly-γ-ethylglutamyl BPA for any gross signs of denaturation: qualitative solubility between pH 2 and 11, appearance of the protein boundary in electrophoresis at pH 8.6, and a mobility estimation at the same pH. Poly-γ-ethylglutamyl BPA was completely soluble throughout the pH range tested, showed a single symmetrical peak in electrophoresis, and a change in mobility which appeared reasonable on the basis of the chemical modification. Thus this derivative showed no obvious signs of denaturation when examined by the above criteria. In contrast, treatment with dilute alkali resulted in a derivative which was insoluble between pH 5 to 2.5, showed at least two components upon electrophoresis, and had a mobility which was much less than that expected on the basis of the charge of the modified protein. It would thus appear that treatment of poly-γ-ethylglutamyl BPA with alkali has resulted in gross structural alteration of the protein. The possibilities of amide or peptide hydrolysis of the modified protein by alkali treatment had not been eliminated, although it does not seem likely that peptide bond hydrolysis would occur to a significant extent (14).

SUMMARY

Poly-γ-ethylglutamyl bovine plasma albumin was prepared by reaction of bovine plasma albumin in phosphate buffer with *N*-carboxy γ-ethyl glutamate anhydride. The molecular weight of the protein calculated from the amount of glutamic acid added was increased from 69,000 to 82,500.

A study of the hydrolysis of modified albumins by proteolytic enzymes revealed that papain and pancreas extracts hydrolyzed poly-γ-ethylglutamyl albumin and polyglycyl albumin to about 75 per cent of that shown with the unmodified protein. With trypsin and chymotrypsin there was a decrease of 55 to 70 per cent for the hydrolysis of the modified albumin preparations as compared to unmodified bovine plasma albumin. However, with pepsin there appeared to be about the same degree of hydrolysis for both the modified and unmodified albumins. A crude peptidase preparation which readily hydrolyzed triglycine had no action on polyglycyl albumin.

BIBLIOGRAPHY

1. Stahmann, M. A., and Becker, R. R., *J. Am. Chem. Soc.*, **74,** 2695 (1952).
2. Becker, R. R., and Stahmann, M. A., *J. Biol. Chem.*, **204,** 745 (1953).
3. Green, M., and Stahmann, M. A., *J. Biol. Chem.*, **197,** 771 (1952).
4. Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, **159,** 333 (1945).
5. Johnson, M. J., Johnson, G. H., and Peterson, W. H., *J. Biol. Chem.*, **116,** 515 (1936).
6. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176,** 367 (1948).
7. Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, **24,** 503 (1916).
8. Maurer, P. H., and Heidelberger, M., *J. Am. Chem. Soc.*, **73,** 2070 (1951).
9. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **178,** 79 (1949).
10. Longsworth, L. G., and Jacobsen, C. F., *J. Phys. and Colloid Chem.*, **53,** 126 (1949).
11. Putnam, F. W., in Neurath, H., and Bailey, K., The proteins, New York, **1,** pt. B, 807 (1953).
12. Alberty, R. A., *J. Chem. Education*, **25,** 426 (1948).
13. Tanford, C., *J. Am. Chem. Soc.*, **72,** 441 (1950).
14. Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, **82,** 171 (1929).

METABOLISM OF NEOPLASTIC TISSUE

VI. ASSAY OF OXIDIZED AND REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE IN NORMAL AND NEOPLASTIC TISSUES*

By LILLIAN A. JEDEIKIN and SIDNEY WEINHOUSE

(From the Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia, Pennsylvania)

(Received for publication, August 16, 1954)

In connection with studies of intermediary metabolism in tumor cells it was desirable to have information concerning the content and the intracellular distribution of diphosphopyridine nucleotide (DPN) and the relative proportions of its oxidized and reduced forms in a variety of normal and neoplastic cell types. Of the large number of analytical procedures currently available (1–4) it was felt that the most desirable method on the basis of reliability, sensitivity, and specificity would be one utilizing a specific enzymatic reaction in which the oxidized and reduced forms of the nucleotide act as oxidant and reductant in the presence of an appropriate dehydrogenase. The system, ethanol-acetaldehyde, appeared to be the reaction of choice, inasmuch as the equilibrium conditions are established (5) and the apoenzyme, alcohol dehydrogenase, is highly specific and readily available (6). Though this reaction has been employed to follow changes in DPN^+ and DPNH in various systems *in vitro* (7–9), it has not to our knowledge been used for the assay of these substances in tissues.[1] The present report describes procedures for the recovery of DPN^+ and DPNH from biological materials under conditions suitable for assay by spectrophotometric means, the Beckman DU spectrophotometer and the ethanol-acetaldehyde system being used. Results are also given of its application to a number of neoplastic and non-neoplastic tissues.

Method

Reagents; Nucleotides—The DPN^+ used as a standard in this study was a product of the Pabst Laboratories. Its purity, determined enzymati-

* Aided by grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, the National Cancer Institute of the Department of Health, Education, and Welfare, and the Atomic Energy Commission, contract No. AT(30-1)777.

[1] Recently, Anderson and Vennesland (10) used alcohol dehydrogenase and glucose-6-phosphate dehydrogenase in conjunction with the spectrophotometer for assay of total DPN and triphosphopyridine nucleotide in green leaves. Owing to low concentrations in this material, a lengthy extraction procedure had to be used, involving adsorption on and elution from charcoal, lyophilization, and precipitation from acetone.

cally, ranged in different preparations from 75 to 83 per cent. DPNH was prepared from the commercial DPN^+ by reduction with hydrosulfite according to the method of Lehninger (8). The purities of three preparations used in this study were 53, 82, and 51 per cent.

Alcohol Dehydrogenase—This was a solution of a recrystallized commercial product from yeast obtained from the Worthington Biochemical Sales Company of Freehold, New Jersey. A solution is made up fresh each day by diluting 1 drop of the original solution to 2 ml.; 0.1 ml. of this diluted solution is sufficient to complete the reaction to a constant optical density in less than 3 minutes. The original solution is stable for many months when kept at 0°.

Substrates—Ethanol and freshly distilled acetaldehyde are diluted with water to a 10 per cent solution by volume. Ethanol solutions are indefinitely stable. Acetaldehyde solutions have not been kept for more than about 2 weeks; they are satisfactory during this period.

Buffers—0.1 M pyrophosphate, pH 9.0. Sodium pyrophosphate, 1.8 gm., is dissolved in water and the solution is adjusted to pH 9.0 by the addition of approximately 0.6 ml. of 1 M HCl with the glass electrode, and is diluted to 40 ml.

Phosphate Buffer, 0.05 M, pH 5.4—50 ml. of a 0.2 M stock solution of KH_2PO_4 are adjusted to pH 5.4 and diluted to 200 ml.

Phosphate Buffer, 0.05 M, pH 8.7—50 ml. of a 0.2 M stock solution of KH_2PO_4 are adjusted to pH 8.7 by addition of approximately 50 ml. of 0.2 M NaOH, and are diluted to 200 ml.

Phosphate Buffer, 0.1 M, pH 7.0—50 ml. of the 0.2 M stock solution of KH_2PO_4 are adjusted to pH 7.0 with 0.2 M NaOH, and are diluted to 100 ml.

Extraction of Nucleotides—The tissues are rapidly excised from the animal which is slaughtered preferably by a method such as decapitation, which allows complete drainage of blood from the large vessels. For DPN^+ assay, approximately 1 gm. is weighed and added immediately to a glass homogenizer tube containing 3 ml. of buffer, pH 5.4, and 1 ml. of water, and which has been previously heated to 100° and maintained at this temperature by immersion in a bath of boiling water. After exactly 1 minute of heating, the tissue is homogenized with a close fitting glass pestle for exactly 30 seconds. The tissue suspension is then chilled in an ice bath and is centrifuged for 10 minutes at 8500 × *g*. Extraction of DPNH is carried out in exactly the same manner, except that the buffer, pH 8.7, is used.

A Servall model SS-1 centrifuge was used in all of our studies, at speeds of approximately 11,000 r.p.m., corresponding to 8500 × *g*. In several trial experiments it was found that centrifuging at higher speeds by using the Spinco model L ultracentrifuge at 20,000 r.p.m. gave clearer supernatant fluids without affecting the yield of nucleotides, but this advantage was not

deemed sufficient to warrant the Spinco centrifuge being used routinely in these assays.

Enzymatic Assay of DPN+—This is conducted with the Beckman model DU spectrophotometer, with the wave-length set at 340 mμ. A 0.5 ml. aliquot of the supernatant solution after centrifugation of the extract, pH 5.4, is mixed in a silica cuvette with 0.1 ml. of ethanol solution, 1.5 ml. of pyrophosphate buffer, pH 9.0, and 0.8 ml. of water. After reading the optical density (Reading 1), 0.1 ml. of enzyme solution is added. When successive readings taken at 1 minute intervals are constant (usually within 3 minutes), Reading 2 is taken, another 0.1 ml. of the enzyme solution is added, and Reading 3 is taken. The difference between Reading 2 and Reading 3 represents corrections both for dilution and for the optical density of the enzyme solution; it is usually about 0.01 to 0.02 unit. This is added to the difference between Readings 2 and 1 to obtain a corrected value for the optical density change due to DPN+. On the basis of a molar extinction coefficient, ϵ = optical density/cl (where optical density = log I_0/I, c = concentration in moles per liter, and l = light path in cm.) of 6.3×10^3 (11), an optical density change of 1.0 is equivalent to 318 γ of DPN+.

DPNH Assay—A 0.5 ml. aliquot of the supernatant solution at pH 8.7 is mixed in the silica cuvette with 0.1 ml. of acetaldehyde solution, 1.5 ml. of buffer, pH 7.0, and 0.8 ml. of water. After reading the density, 0.1 ml. of the enzyme solution is added and the same procedure is followed as described for DPN+ estimation. In this instance, the difference between Readings 2 and 3 is subtracted from the difference between Readings 1 and 2 to obtain the corrected optical density due to DPNH.

For both estimations considerable leeway is permissible. Larger or smaller aliquots of the extracts may be taken with correspondingly less or more water. Since optical density changes of 0.002 are readily detectable, the estimation of about 15 γ of nucleotide, with an optical density change of 0.047, should have a probable error of 5 per cent. The DPN extracts are stable for days when kept at 0°. However, the DPNH extracts lose about 25 per cent in 24 hours; hence it is advisable to assay these the same day they are prepared.

EXPERIMENTAL

Extraction of DPN+ and DPNH—When cell structure is destroyed, DPNases, previously inactive or inaccessible, rapidly break down DPN+ and DPNH. Any extraction procedure consequently requires inactivation or destruction of these enzymes. This may be done by extracting at very low temperatures (3, 12), by adding inhibitors (4), or by heating with acids or bases (1, 2, 13, 14). None of these procedures was regarded as entirely

satisfactory for the spectrophotometric procedure we had in mind, since they involved use of enzyme poisons or yielded turbid suspensions of high and unstable optical densities. The use of heat to inactivate DPNases was originally employed by von Euler and Schlenk (13) to isolate DPN^+ and has been employed subsequently in connection with many isolation and analytical procedures (1, 2). Though the enzymes responsible for pyridine nucleotide destruction are effectively destroyed by heating, the nucleotides themselves are notably labile at high temperatures, particularly in certain pH ranges. It has long been known that, whereas, on heating, DPN^+ is more stable in acid solutions than in alkali, DPNH exhibits the opposite

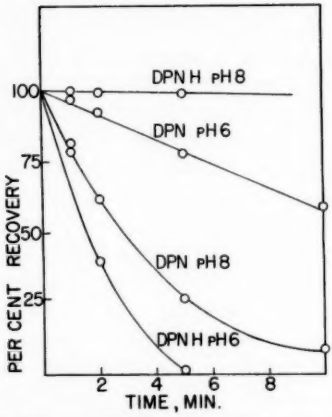


FIG. 1. Recovery of DPN^+ and DPNH after heating at 100° for various periods. Values are given in per cent of the initial value obtained without heating.

behavior. This phenomenon has in several instances been employed for the differential destruction of one form in the presence of the other (2, 13, 14).

Since this heating procedure appeared to offer the best means of obtaining tissue extracts suitable for enzymatic estimation of DPN^+ and DPNH, a detailed study was made of the stability of these nucleotides at 100° under various conditions. Our original hope was that a single set of conditions might be found, possibly at a pH near neutrality, at which these substances would be sufficiently stable to enable the recovery of both in a single extraction. However, this approach was abandoned when it became evident that the stabilities of both forms are highly pH-dependent. The extraordinary lability of DPN^+ and DPNH at 100° is illustrated in Fig. 1. Here a mixture of the two nucleotides was heated in buffers, either at pH 6.0 or 8.0, and samples were withdrawn after 1, 2, 5, and 10 minutes. These were

then assayed by the enzymatic procedure described earlier. Whereas at pH 6 only 7 per cent of the DPN^+ was destroyed in 2 minutes, 38 per cent was destroyed in the same time at pH 8. On the other hand, DPNH is stable at pH 8, but is 60 per cent destroyed in 2 minutes at pH 6. These results indicated that by using two extractions, one at pH 6 and one at pH 8, reasonably complete recoveries of both nucleotides could be accomplished. No attempt was made to extend the pH values of the extraction fluids to more acid and alkaline ranges. Since the enzymatic assays require considerable alteration of the pH of these extracts, it was felt advisable to maintain the pH of the extracts as close to neutrality as was consistent with the stabilities of the nucleotides. Satisfactory recoveries of added DPN^+ and DPNH were obtained by using extracting fluids of pH 5.4 and 8.7 for DPN^+ and DPNH, respectively. The high buffering capacity of the tissues brings the pH of the final extracts to 6.3 and 7.7.

Originally tissues were homogenized immediately upon addition to the boiling buffer solution. Occasional low recoveries or inconsistent results led us to suspect that under these circumstances the cell structure was destroyed more rapidly than the DPNases, and appreciable enzymatic destruction of the nucleotides may have been occurring during homogenization. This factor was avoided, and consistently high recoveries were secured by keeping the tissue at 100° for 1 minute before homogenization, thus making certain that DPNases are destroyed before breaking up the cells. The data on the pure coenzymes shown in Fig. 1 indicated that longer heating periods might be employed without undue destruction, but tests of the stability of tissue DPN^+ and DPNH indicated that they are less stable than anticipated from the data obtained with the pure coenzymes. For example, in the experiment shown in Table I the DPN^+ assay of rat liver was lowered 34 per cent by heating 4 minutes and that of DPNH was lowered 58 per cent. The great thermal lability of tissue DPNH has been repeatedly observed, and we regard it as important to avoid heating periods of more than 1 minute in the assay of this nucleotide.

Stability of Pyridine Nucleotides in Intact Tissues in Situ—To obtain some idea of the magnitude of changes in DPN^+ and DPNH in tissues during the time required for sacrifice of the animal and dissection of the tissue, the following experiment was performed. A rat was decapitated, the abdomen was opened, and portions of the liver were excised immediately and after standing for 1, 2, and 4 hours at room temperature. As shown in Table II, there was a gradual decrease in DPN^+, amounting to 60 per cent in 4 hours. A similar decrease in DPNH was also observed. The apparent stability of DPNH during the 1st hour is probably due to a conversion of DPN^+ to DPNH as a result of tissue anaerobiosis. Similar results were obtained in a second experiment in which the liver was immediately excised and samples

were analyzed after 1 and 2 hours standing at room temperature. These results thus indicate that large variations in the amounts or proportions of the two forms of the nucleotide would not be expected during the 5 or 10 minutes ordinarily required for removal of tissues.

DPN⁺ and DPNH Content of Tissues—In Table III there is recorded a survey of various animal tissues with respect to DPN⁺ and DPNH content. The values are regarded as minimal, but probably close to the actual, since

TABLE I

DPN⁺ and DPNH Content of Rat Liver Heated to 100° for Various Periods

Tissue was heated for the time indicated, then homogenized for ½ minute and cooled. The values are in micrograms per gm. of fresh tissue.

Heating time	DPN⁺ at pH 6	DPNH at pH 8
min.		
1	411	318
2	340	210
4	270	134

TABLE II

Postmortem Stability of Tissue DPN⁺ and DPNH in Situ

The values are in micrograms per gm. of fresh tissue.

Time after sacrifice	Experiment 1		Experiment 2	
	DPN⁺	DPNH	DPN⁺	DPNH
hrs.				
0	331	200	398	254
1	235	198	330	216
2	169	134	267	188
4	132	86		

the addition of DPN⁺ and DPNH to tissues before homogenization resulted in recoveries consistently in the vicinity of 85 per cent. A closer approximation to the true value might thus be attained by increasing the values by about 18 per cent.

Nucleotide content was high in liver, heart, kidney, and muscle, intermediate in brain and spleen, and lowest in blood. No marked species differences were observed. In general, the values for total DPN given in Table III are within the magnitude of values reported by previous investigators (1, 2, 4, 12–17). However, they are considerably below the high DPN levels found in rat liver by Jandorf (18) and in liver, kidney, and heart by Axelrod and Elvehjem (19). In both studies procedures were employed involv-

ing yeast fermentation but with entirely different methods of extraction. Robinson *et al.* (20) also reported values of DPN in rat liver and kidney which were considerably higher than ours, but the fluorometric and bacterial assay procedures employed by them are probably not entirely specific for the intact nucleotide.

TABLE III

DPN⁺ and DPNH Content of Tissues

The values are in micrograms per gm. of fresh tissue.

Tissue	DPN⁺			DPNH			Total	
	No. of determinations	Average	Range	No. of determinations	Average	Range	Average	Range
Rat liver	6	373	338–411	8	315	254–372	688	592–783
Mouse liver	5	517	394–592	4	156	57–204	673	451–796
Pigeon "	2	425	404–446	2	175	159–191	600	563–637
Rabbit "	1	406		1	156		562	
Rat kidney	2	286	276–296	2	182	175–188	468	451–484
Mouse kidney	2	429	423–435	2	232	199–264	661	622–699
Pigeon "	2	243	206–280	2	156	137–175	399	343–455
Rabbit "	1	216		1	162		378	
Rat heart	2	476	474–477	2	87	65–108	563	539–585
Mouse heart	2	397	378–415	2	271	270–272	668	648–687
Pigeon "	2	366	334–398	2	46	41– 51	412	375–449
Rabbit "	1	140		1	105		245	
Rat spleen	2	178	162–194	2	41	37– 44	219	199–238
" skeletal muscle	2	354	352–356	2	16	10– 22	370	362–378
Mouse skeletal muscle	2	357	344–370	2	16	16– 16	373	360–386
Pigeon breast muscle	2	580	530–630	2	33	27– 38	613	557–668
Rabbit skeletal muscle	1	451		1	19		470	
Rat brain	2	107	102–111	2	91	80–102	198	182–213
Mouse brain	2	193	181–204	2	105	95–114	298	276–318
Rat whole blood	1	45		1	13		58	

Assay values for several neoplastic cell types are given in Table IV. Total DPN content was considerably below that found in most of the normal tissues, but was close to levels observed in spleen and brain. In contrast with the wide range in total DPN of normal tissues, the solid tumors displayed a narrow range of between 118 and 172 γ per gm. It is of interest that oxidative activity toward components of the citric acid cycle displayed by these same tumors also covered a narrow range similar in magnitude to that of the less highly active normal tissues (21). Considerably

higher DPN levels were found in two ascites tumors, but these may reflect their lack of connective tissue rather than a real difference in cell composition. The low DPN content of tumors has also been observed by others (3, 4, 14, 17, 22).

Ratio of Oxidized and Reduced DPN—A feature of the present study was the consistent preponderance of DPN^+ over DPNH in all tissues examined. Rat liver displayed a consistently low and a relatively constant ratio of DPN^+-DPNH, ranging in six analyses from 1.05 to 1.4. Livers of other species gave higher ratios, however, varying from 2.6 to 6.9. Intermediate values for the ratio were observed in such normal tissues as brain, heart,

TABLE IV

DPN^+ and DPNH Content of Tumors

The values are given in micrograms per gm. of fresh tissue.

Tumor	DPN^+			DPNH			Total	
	No. of determinations	Average	Range	No. of determinations	Average	Range	Average	Range
Hepatoma 98/15	2	103	92–114	2	37	35–38	140	127–152
Rat hepatoma	2	248	200–296	1	51		299	
Rhabdomyosarcoma	2	109	99–118	2	24	19–29	133	118–147
Sarcoma 37	2	105	89–121	2	42	33–51	147	122–172
Mouse mammary carcinoma	2	105	99–111	2	39	37–41	144	136–152
Ascites sarcoma 37	1	206		1	32		238	
Ehrlich's ascites carcinoma	1	297		1	0		297	

kidney, and spleen, but very high values ranging to over 20 were invariably observed in skeletal muscle of various species. Again a variety of tumors gave ratios varying within the relatively narrow range of 2.5 to 4.5.

Published assays of tissue DPN^+ and DPNH are relatively few. Von Euler *et al.* (23), using differential destruction of each form with acid or alkali, coupled with a fermentation assay, reported that, whereas rat muscle had somewhat more DPN^+ than DPNH, there was a 4- to 5-fold higher level of the reduced form in the Jensen sarcoma. Using a similar procedure, Fisher and Schlenk (14) reported ratios in several rat and mouse tumors not far different from those of the present study, though the levels of total nucleotide were considerably below ours. Bonsignore (24), using a fluorometric assay, found twice as much DPNH as DPN^+ in rat liver.

The ratio of the oxidized and reduced forms of DPN and triphosphopyridine nucleotide was studied by Feigelson *et al.* (12). Frozen tissues were

homogenized with 2 per cent trichloroacetic acid and the pyridine nucleotides adsorbed on charcoal. Total nucleotide content was determined by oxidizing the reduced form of the coenzymes in the extract with hydrogen peroxide and assaying spectrophotometrically by reduction with sodium hydrosulfite. Reduced coenzymes were determined by the difference between a peroxide-treated and untreated extract. Rat liver was found to contain 631 γ of DPN^+ and 204 γ of DPNH per gm. of tissue. These values are similar to those reported by us for this tissue.

DISCUSSION

Until a better idea is obtained of factors which might affect the ratio of DPN^+ to DPNH in tissues, any conclusions concerning the significance of this ratio are unwarranted. It is already evident, however, that tumors as a class do not display marked deviations in this ratio from normal tissues; on the contrary, they exhibit a rather narrow range, which is well within a much wider range given by non-neoplastic tissues. One factor which could conceivably influence the ratio is the balance between substrates which require DPN^+ for their oxidation and electron acceptors comprising the DPNH-oxidation system (the flavins and cytochromes). The present results suggest, on this basis, that this balance is essentially similar in both tissue types and are not in accord with previous suggestions that tumors as a class are deficient in electron transport factors (25). On the other hand the content of total DPN is considerably lower in tumors than in the more active normal tissues, and this property deserves consideration as a possible factor in some of the metabolic peculiarities of the cancer cell. Again, however, other normal tissues also have low DPN levels, *e.g.* brain and spleen; hence this is not a unique characteristic of the cancer cell. Further comparisons are under way concerning the intracellular distribution of DPN^+ and DPNH, and the activity and distribution of enzymes which split these substances.

SUMMARY

A method is described for the assay of DPN^+ and DPNH in normal and neoplastic tissue involving extraction with hot buffer solutions, followed by spectrophotometric determination of each nucleotide, with alcohol apodehydrogenase and the appropriate substrate.

Total DPN content was high in liver, kidney, and muscle of the rat, mouse, rabbit, and pigeon, lower in spleen and brain, and lowest in blood. The DPN contents of a variety of transplanted tumors were in the range of spleen and brain. Ratios of DPN^+ to DPNH were invariably greater than 1, ranging from about 1.2 in rat liver to over 20 in skeletal muscle. Tumors displayed intermediate values of this ratio of 2.5 to 4.5.

BIBLIOGRAPHY

1. Singer, T. P., and Kearney, E. B., *Advances in Enzymol.*, **15,** 79 (1954).
2. Schlenk, F., in Sumner, J. B., and Myrbäck, K., The enzymes, **2,** 254 (1952).
3. Carruthers, C., and Suntzeff, V., *Arch. Biochem. and Biophys.*, **45,** 140 (1953).
4. Strength, D. R., Ringler, I., and Nelson, W. L., *Arch. Biochem. and Biophys.*, **48,** 107 (1954).
5. Racker, E., *J. Biol. Chem.*, **184,** 313 (1950).
6. Pullman, M. E., Colowick, S. P., and Kaplan, N. O., *J. Biol. Chem.*, **194,** 593 (1952).
7. Kornberg, A., and Lindberg, O., *J. Biol. Chem.*, **176,** 665 (1948).
8. Lehninger, A. L., *J. Biol. Chem.*, **190,** 345 (1951).
9. Burton, K., and Wilson, T. H., *Biochem. J.*, **54,** 86 (1953).
10. Anderson, D. G., and Vennesland, B., *J. Biol. Chem.*, **207,** 613 (1954).
11. Drabkin, D. L., *J. Biol. Chem.*, **157,** 563 (1945).
12. Feigelson, P., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, **185,** 741 (1950).
13. von Euler, H., and Schlenk, F., *Z. physiol. Chem.*, **246,** 64 (1937).
14. Fisher, A., and Schlenk, F., *Texas Rep. Biol. and Med.*, **6,** 346 (1948).
15. von Euler, H., Schlenk, F., Heiwinkel, H., and Högberg, B., *Z. physiol. Chem.*, **256,** 208 (1938).
16. Lutwak-Mann, C., *Biochem. J.*, **32,** 1364 (1938).
17. Carruthers, C., and Suntzeff, V., *Cancer Res.*, **14,** 29 (1954).
18. Jandorf, B. J., *J. Biol. Chem.*, **150,** 89 (1943).
19. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **131,** 77 (1939).
20. Robinson, J., Levitas, N., Rosen, F., and Perlzweig, W. A., *J. Biol. Chem.*, **170,** 653 (1947).
21. Weinhouse, S., Millington, R. H., and Wenner, C. E., *Cancer Res.*, **11,** 845 (1951).
22. Bernheim, F., and Felsovanyi, A. V., *Science*, **91,** 76 (1940).
23. von Euler, H., Malmberg, M., and Gunther, G., *Z. Krebsforsch.*, **45,** 425 (1937).
24. Bonsignore, A., *Boll. Soc. ital. biol. sper.*, **24,** 939 (1948).
25. Greenstein, J. P., Biochemistry of cancer, New York, 2nd edition, 364 ff. (1954).

THE BIOSYNTHESIS OF CHOLINE FROM SERINE AND RELATED COMPOUNDS*

By DAVID ELWYN,† ARTHUR WEISSBACH,‡ SARAH S. HENRY, AND DAVID B. SPRINSON

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York)

(Received for publication, August 18, 1954)

The reactions whereby the ethanolamine and methyl groups of choline are synthesized from serine and glycine in the rat have been briefly reported earlier (1). In the synthesis of the methyl groups from serine-β-$C^{14}D_2OH$, it was further shown that carbon-hydrogen bonds are not broken (2). This suggested that if a compound analogous to 5-formyl-5,6,7,8-tetrahydrofolic acid participates in the transfer of C_1 intermediates the substituent on N-5 might be capable of existence in a more reduced form. The possible effect of selective cleavage of C—H bonds on the validity of the results was investigated and shown to be unimportant. The present report contains a more complete description of our findings.

EXPERIMENTAL

Synthesis of Labeled Compounds[1]

DCOOH—127 gm. (1.96 moles) of dry KCN, 200 gm. (10 moles) of D_2O, and 4 gm. (0.1 mole) of NaOH were heated for 5 hours at 175° in bomb tubes (3). After removal of excess D_2O by distillation the residue was dissolved in water, acidified with 100 ml. (1.8 moles) of concentrated H_2SO_4, and continuously extracted with three successive portions of alcohol-free ether for three 6 hour periods. Distillation of the ether through a Vigreux column left 1.91 moles of formic acid as a 70 per cent aqueous solution.

* This work was supported by grants from the American Cancer Society (on recommendation of the Committee on Growth of the National Research Council), the Lederle Laboratories Division of the American Cyanamid Company, the National Institutes of Health, United States Public Health Service, and the Rockefeller Foundation.

† Postdoctoral Fellow of the Life Insurance Medical Research Fund, 1950–52. Present address, Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts.

‡ Postdoctoral Fellow of the Life Insurance Medical Research Fund, 1952–53. Present address, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Maryland.

[1] D_2O and C^{14} were obtained on allocation from the United States Atomic Energy Commission.

DC^{14}OONa—12.4 mmoles of DCOOH were neutralized with NaOH and mixed with 0.12 mmole of HC^{14}OONa obtained from Tracerlab. An aliquot was diluted with normal HCOONa and dried thoroughly for analysis of D. C^{14} activity was determined on $BaCO_3$ obtained by treatment of the formate solution with $HgCl_2$.

Deuterioformaldehyde—51 gm. (0.34 mole) of anhydrous dihydroxymaleic acid (dried to constant weight *in vacuo* at 78° over P_2O_5) were heated[2] for several hours at 70° in 300 ml. of 99.8 per cent D_2O until evolution of CO_2 had ceased (4). In order to convert the resulting glycolaldehyde to formaldehyde, a solution of 68.4 gm. (0.3 mole) of periodic acid in 50 ml. of H_2O was added to the cooled (10°) reaction mixture. After 20 minutes, 40 gm. (0.15 mole) of $SrCl_2$ were added, the solution was adjusted to pH 6 with 1 N NaOH, and, following refrigeration overnight, insoluble strontium salts were removed by filtration. The filtrate was distilled at atmospheric pressure until 40 ml. remained and then steam-distilled until a total volume of 500 ml. of distillate had been collected. The yield of deuterioformaldehyde, as determined by bisulfite titration, was 0.11 mole (33 per cent).

L-*Serine-3-C^{14},2,3-D,N^{15}*—This compound, Serine I, was described previously (5). (For Serine I-B, it was diluted with L-serine-2,3-D, prepared (5) by reduction of ethyl formyl hippurate in ethereal solution with aluminum amalgam and D_2O.) Serine so made contained 55 per cent of its deuterium (6) in the β position.

L-*Serine-3-C^{14},3-D*—1 mole of DCOOH and 0.8 mmole of HC^{14}OONa (0.8 mc.) were dissolved in 1.5 liters of absolute ethanol. A few drops of concentrated H_2SO_4 were added, and the mixture was distilled under atmospheric pressure through an efficient glass helix column. The main portion of the ethyl formate distilled at 52–54°. The fraction boiling at 38–57° was treated with sodium ethoxide and ethyl hippurate (7, 8) in absolute alcohol; considerable exchange took place with the hydrogen of the solvent,[3] leaving only 15 per cent of the D of the formic acid in the ethyl formyl hippurate. This by reduction in ethereal solution with H_2O and aluminum amalgam gave, after hydrolysis, 12.5 gm. of DL-serine-3-C^{14},3-D (12 per cent based on formic acid). After resolution of the *N*-chloroacetyl derivative with Armour acylase (9), 3.05 gm. of L-serine-3-C^{14},3-D (Serine II) were obtained (49 per cent).

[2] It is of interest that dihydroxymaleic acid decarboxylates readily in H_2O at 50°, but not noticeably in D_2O at this temperature (Elwyn, D., Henry, S. S., and Sprinson, D. B., unpublished observations).

[3] Extensive labilization of D (50 per cent) was observed when ethyl deuterioformate was added to sodium ethoxide in ethanol and reisolated in 2 hours (Rothschild, J., Elwyn, D., and Sprinson, D. B., unpublished results).

$C_3H_7O_3N$ (105.2). Calculated, N 13.3; found, N 13.2
$[\alpha]_D^{23}$ $-7.0° \pm 0.2°$ (8.07% in H_2O)

L-*Serine-3-C*^{14},*3-D*$_2$—Approximately 1 mmole (0.5 mc.) of formaldehyde-C^{14} (obtained from the Oak Ridge National Laboratory) was added to the solution of 0.11 mole of CD_2O (described earlier) and treated with a slight excess of ethyl acetamidomalonate (10). The resulting serine was resolved enzymatically via the *N*-chloroacetyl derivative. Considerable difficulties were encountered in the resolution owing to the presence of glycine, which was removed by chromatography on Dowex 50 with 1.5 N HCl. The yield of twice recrystallized L-serine-3-C^{14},3-D_2 (Serine III) was 590 mg. (10 per cent based on formaldehyde).

$C_3H_7O_3N$ (106.5, corrected for D). Calculated. C 33.8, H 8.0, N 13.2
Found. " 33.8, " 7.8, " 13.2
$[\alpha]_D^{25} = -7.3° \pm 0.2°$ (6.7% in water)

Labeled Glycines—Glycine-2-C^{14} was prepared from $C^{14}O_2$ by the reaction series $CO_2 \rightarrow CH_3OH \rightarrow CH_3I \rightarrow CH_3CN \rightarrow CH_3COOH \rightarrow CH_3COBr \rightarrow BrCH_2COBr \rightarrow BrCH_2COOH \rightarrow CH_2NH_2 \cdot COOH$ (11–15). Glycine-1-C^{14} was the kind gift of Dr. D. Rittenberg (16). Glycine-N^{15} was prepared by the phthalimide method (17).

Isotope Analyses

C^{14} activity was measured with a thin window, or internal flow, Geiger-Müller counter on the compounds isolated or, when CO_2 was obtained, on $BaCO_3$. Stainless steel or Teflon dishes of 1 or 2 sq. cm. area were used, and samples were counted at, or corrected to, "infinite" thickness. Back-scattering corrections were 1.5 for choline chloride·$6HgCl_2$, 1.2 for $BaCO_3$ and tetramethylammonium iodide, and 1.15 for ethanolamine diiodosalicylate. Unless otherwise noted, sufficient counts were taken to give a standard error of less than 5 per cent. Radioactivity is expressed as counts per minute per dish (2 sq. cm.) × molecular weight divided by 12.

N^{15} (18) and D (19) concentrations were measured by the usual methods. "Atoms excess D" per molecule is (atom per cent excess D/100) × (number of hydrogen atoms in the compound).

Administration of Compounds

The test substances were fed to adult male white rats in 8 to 10 gm. of a 15 per cent casein diet (20) made into a paste with a little water. The activities and the quantities of the materials administered are reported in Table I.

One sample of choline was isolated from the lipides of 24 day-old rats

which were *injected* with Serine I. The details have been described elsewhere (21).

Isolation of Choline and Ethanolamine

The combined internal organs (liver, spleen, lungs, heart, kidneys, testes, and washed small intestine) were homogenized in absolute alcohol in a Waring blendor. After removal of the solvent by filtration the tissues

TABLE I

Administration of Test Substances to Rats

Compound	Isotope concentration			Duration	No. of rats	Total weight of rats	Amount fed per 100 gm.
	C^{14}	D	N^{15}				
	c.p.m. × 10^{-4}*	*atoms excess*	*atoms per cent excess*	*days*		*gm.*	*mmoles*
L-Serine-3-C^{14},2,3-D,N^{15} (I-A)	32.8	1.29†	25.8	2	4	1050	0.94
" (I-B)	6.56	1.12†	5.17	2	4	900	1.05
" (I-C)‡	32.8	1.29†	25.8	5	20	760	1.52
L-Serine-3-C^{14},3-D (II)	13.1	0.13		2	4	670	1.14
L-Serine-3-C^{14},3-D_2 (III)	38.0	1.41		2	4	680	0.56
Sodium formate-C^{14},D	115	0.99		1	4	710	1.7
Glycine-2-C^{14},N^{15}	342		20.6	1	4	1000	0.40
Glycine-1-C^{14},N^{15}	10.9		27.6	2	2	550	2.9

* Counts per minute under standard conditions × the molecular weight of the compound divided by 12.

† 55 per cent of the deuterium was in the β position.

‡ Injected intraperitoneally; the rats were 18 days old at the start of the experiment (21). A total of 10 mmoles of serine was administered at the rate of 0.1 mmole per rat per day. The weight gain over a 5 day experimental period was from 560 to 760 gm. The dose reported in the last column is based on a median weight of 660 gm.

were extracted with hot absolute alcohol (8 hours) and 2:1 hot alcohol-ether (8 hours). The residue which remained after removal of the solvents by distillation *in vacuo* was taken up in chloroform, filtered, and, after removal of chloroform, hydrolyzed by refluxing for 5 hours with 6 N methanolic HCl (22). The hydrolysate was evaporated to dryness and distributed between ether and water. The aqueous phase was evaporated to dryness, taken up in water, treated with 5 per cent neutral lead acetate (23) to complete precipitation, and centrifuged. The supernatant fluid was acidified with HCl, treated with H_2S, filtered, and the filtrate taken to dryness. The residue was taken up in absolute alcohol, the solution clarified by centrifuging, and then treated with a saturated solution of

$HgCl_2$ in absolute alcohol. The choline chloride·$6HgCl_2$ was removed by centrifugation, washed with 2 per cent aqueous $HgCl_2$, and recrystallized to constant activity from the same solution. Yield from 1 kilo of rats, about 1.7 gm.

Choline chloroplatinate was prepared from the $HgCl_2$ complex by removing Hg^{++} with H_2S and adding a 1 M solution of chloroplatinic acid in ethanol to a solution of the choline chloride in a small volume of the same solvent. Removal by centrifugation or filtration yielded products (m.p. 218–220°) which had activities close to those of the $HgCl_2$ complexes. A typical analysis follows.

$C_{10}H_{28}O_2N_2Cl_6Pt$ (616.3).	Calculated.	C 19.5, H 4.6, Pt 31.7
	Found.	" 19.1, " 4.9, " 32.0

For the isolation of ethanolamine the supernatant solution from the choline-$HgCl_2$ complex was treated with H_2S and filtered. An excess of an aqueous suspension of $Ca(OH)_2$ was added to the filtrate and ethanolamine distilled *in vacuo* (bath temperature 80°) into dilute HCl. This solution was made alkaline with NaOH and extracted continuously with ether. An excess of diiodosalicylic acid was placed in the solvent flask (24). The precipitated ethanolamine diiodosalicylate was recrystallized from alcohol-petroleum ether; m.p. 198–202°.

Degradation of Choline

C^{14}:D Ratio of Methyl Groups—Trimethylamine, isolated as the chloroplatinate, was obtained from the choline salt by gentle boiling in alkaline $KMnO_4$ (25). Analysis of the trimethylamine salt gave the C^{14} and D of the methyl groups. The activity of the $BaCO_3$ obtained during the determination of D always agreed with that of the chloroplatinate. A typical elementary analysis follows.

$C_6H_{20}N_2Cl_6Pt$ (528.3).	Calculated.	C 13.7, H 3.8, Pt 37.0
	Found.	" 13.2, " 4.0, " 37.4

Activity of C-1, C-2, and CH_3^- of Choline—In order to obtain separately the activities of C-1 (hydroxyl carbon) and C-2 of the ethanolamine moiety of choline, the base derived from the $HgCl_2$ complex was first oxidized to betaine with acid $KMnO_4$ (26). The betaine hydrochloride (yield, 75 to 85 per cent) was contaminated with variable amounts (5 to 10 per cent) of KCl, but after correction for ash gave satisfactory elementary analyses. A typical example is given below.

$C_5H_{12}O_2NCl$ (153.5).	Calculated.	C 39.1, H 7.8
	Found (corrected for 5.13% ash).	C 39.5, H 8.3

Labeled betaine hydrochloride was diluted (if necessary) with normal

material to approximately 50 mg. and added to a mixture of 1 gm. of diphenylamine and 1 gm. of diphenylmethane. After removal of extraneous CO_2 at 50–80° in a stream of N_2, the mixture was heated to 270–290° in a Woods metal bath (27). Trimethylamine was removed in a dilute H_2SO_4 scrubber, and CO_2 was collected in baryta. The $BaCO_3$ represents C-1. Yield, 40 to 50 per cent.

Another sample of approximately 100 mg. of betaine hydrochloride was heated at 160–180° with 10 gm. of KOH and 1 ml. of H_2O. A stream of N_2 was passed through the melt for 4 hours, and trimethylamine was absorbed in two traps containing a concentrated solution of methyl iodide in absolute alcohol. After evaporation to dryness the tetramethylammonium iodide was recrystallized from absolute alcohol. Yield, 30 to 40 per cent. It is possible that degradation of betaine with alkaline permanganate (25) would have produced better yields.

Trial degradations with synthetic betaine-2-C^{14} showed that the CO_2 resulting from decarboxylation in diphenylamine-diphenylmethane was inactive, but that the trimethylamine produced in this reaction had considerable activity. On the other hand, trimethylamine obtained in the alkali fusion was inactive.

The activity of C-2 may be calculated from the values of total choline, trimethylamine, and C-1. This calculated value is significant only when the activity of C-2 is large in comparison with the activities of the methyl groups and C-1, *e.g.* after administration of glycine-2-C^{14}. It is not significant in the experiments in which serine-3-C^{14} was fed. It was useful, whenever material sufficed, to determine the D content of the betaine hydrochloride. When the administered serine was not labeled in the α position with D, this analysis gave, by difference from choline, the D on C-1, as well as a check on the value for D in the methyl groups.

RESULTS AND DISCUSSION

It was pointed out in our preliminary communication (1) and is shown in greater detail here that serine and biologically related compounds are precursors of both the ethanolamine moiety and the methyl groups of choline. This was also observed, under different experimental conditions, and discussed by Arnstein (28). Incorporation of glycine (23) and serine (29) nitrogen into ethanolamine and choline was first found by Stetten.

Ethanolamine Moiety of Choline—The utilizations of the amino group of serine for the nitrogen of the ethanolamine moiety, and of the β-carbon of serine for C-1, are approximately the same (Table II). Whenever betaine was analyzed for D (not shown in Table II) to give by difference from choline the deuterium concentration on C-1, the utilizations of β-D and β-C^{14} for C-1 were also observed to be the same. The similarly equal

utilization of β-C^{14}, β-D, and N^{15} from administered serine for serine of internal organ proteins (6) indicates that ethanolamine is derived as a unit from the serine molecule. In this, the synthesis of choline resembles the utilization of C-3, C-2, and N of serine for C-1, C-2, and N, respectively, of sphingosine (21). But, whereas either serine or ethanolamine (23) can be used for choline synthesis, only serine is used for sphingosine formation.

TABLE II

Utilization of C^{14}, N^{15}, and D of Various Precursors for Choline Formation

Precursor*	Isotope concentration† in choline						Isotope dilution in methyl groups	
	Total			C-1	Trimethylamine			
	C^{14}	D	N^{15}	C^{14}	C^{14}	D	C^{14}	D
	c.p.m.	*atom excess*	*atom per cent excess*	*c.p.m.*	*c.p.m.*	*atom excess*		
Serine I-A	14,000	0.0296	0.277	3400	10,000	0.0195	33	36
" I-B	2,270	0.0235	0.064	800	1,460	0.0123	45	50
" I-C	6,950	0.0169	0.299	4350	2,600	0.0059	126	120
" II	3,940	0.0039		1990	1,950	0.0024	67	54
" III	5,370	0.0186		2250	3,040	0.0103	125	137
$DC^{14}OONa$	31,700	0.0302			21,750	0.0197	53	50
Glycine-2-C^{14},N^{15}	10,750		0.041	1350‡	4,500			
Glycine-1-C^{14},N^{15}	0		0.430					

* See Table I for details. The β-D of Serines I-A, I-B, and I-C was 0.71, 0.615, and 0.71, respectively.

† C^{14} activity in counts per minute under standard conditions × the molecular weight of the compound divided by 12. The molecular weights of salts of dibasic acids, *e.g.* choline chloroplatinate, were divided by 24. Atom excess D = (atom per cent excess D/100) × (number of hydrogen atoms in molecule). See "Experimental" for actual compounds analyzed and degradation procedures.

‡ By difference, C-2 is 4900 c.p.m. The dilution of N^{15} from glycine-2-C^{14},N^{15} is 502; that of C^{14} for C-2 is 684. The resulting ratio of dilutions is close to that found in N^{15} and α-C^{14} of serine derived from the same precursor (6).

The inability of serine betaine to replace choline as a source of lipotropic methyl groups (30) suggests that decarboxylation of serine precedes methylation. It will be shown below that conversion of serine to ethanolamine is an extensive metabolic pathway for this amino acid.

The incorporation of glycine and formate into ethanolamine appears to involve their prior conversion to serine. After the administration of glycine-2-C^{14},N^{15} the ratio of activities of C-1 to C-2 in choline is close to that of C-3 to C-2 found in the internal organ serine of the same animals (6); similarly, the ratio of label of C-2 to N^{15} in choline is also close to that of

serine (6). As in the synthesis of sphingosine, the carboxyl group of glycine is not used. The utilization of formate for ethanolamine synthesis corresponds to its known condensation with glycine to yield serine (31).

Synthesis of Methyl Groups of Choline—It is clear from the results of Table II that the synthesis of choline exemplifies the ability of serine to dissociate not only by decarboxylation, but also by cleavage to glycine (32) and a C_1 fragment (5). This fragment is very effectively converted into the methyl groups of choline as well as the methyl of thymine and C-2 and C-8 of purines (5, 33). Formate (28, 34, 35) and the α-carbon of glycine (1, 33, 36) are also utilized for these positions. The following discussion will be limited to several unique aspects of the conversion of the β-carbon atom of serine to methyl groups.

The findings recorded in the last two columns of Table II show that in this conversion of serine, labeled with both C^{14} and D in the β position, the C^{14}:D ratio is substantially unaltered. This constancy is even more striking than that found in the methyl group of thymine (33). The higher deuterium dilutions in the latter may be due, in part, to the availability of only small amounts of pyrimidine for analysis. For comparison, an experiment with $DC^{14}OONa$ was performed, and the equal utilization of D and C^{14} for methyl groups was confirmed (37). Methyl group transfer from methionine to choline also proceeds without cleavage of C—H bonds (38).

Abundant evidence is available that folic acid is involved in the metabolism of C_1 fragments which are characterized by a lower level of oxidation than that of CO_2. Included in these reactions are the cleavage (39) and synthesis of serine (40) and the synthesis from the β-carbon of serine of the methyl groups of choline (41). Although pteroylglutamic acid is nutritionally effective as folic acid in higher animals and several strains of bacteria, a metabolically related form of this compound which occurs in natural materials is required for the growth of *Leuconostoc citrovorum* (42). The isolation of this form of the vitamin from liver (43) and its identification as 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (44) suggested that it served as a carrier of these fragments in at least one stage of their metabolism. The "active" C_1 intermediate was assumed to participate catalytically in condensation or cleavage reactions in a manner analogous to that of acetyl coenzyme A.

A formylated coenzyme could be reasonably postulated in the synthesis of purines (carbons 2 and 8), or in the utilization of imidazole C-2 of histidine (45), indole α-carbon of tryptophan via N'-formyl-L-kynurenine (46), and administered formate. However, the observation that C—H bonds are not broken in the over-all conversion of the hydroxymethyl group of serine to methyl indicates that formate or formyl derivatives of folic acid are not obligatory intermediates in this process.

These results do not exclude other derivatives of folic acid as coenzymes. The postulation of an *N*-5-hydroxymethyl, or closely related, derivative of tetrahydrofolic acid would be in agreement with the observed stability of the β-hydrogen atoms of serine. This stability is further illustrated by the unaltered C^{14}:D ratio of internal organ serine following the administration of serine-β-$C^{14}D_2OH$ (6). In the experiments reported in Table II the C^{14}:D ratios of the visceral protein serine (not shown in Table II) were also the same as those in the labeled serine administered, even though an extensive cleavage and resynthesis of serine must have been going on. Similar results were obtained in the conversion of $DC^{14}OONa$ to serine. When either labeled serine or formate was given, the deuterium of C-2 and C-8 of purines, however, was found to be extensively labilized (33). It

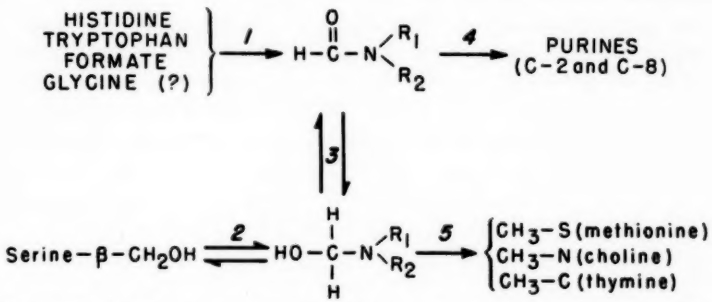


FIG. 1. $R_1(R_2)N$— represents a biologically active form of folic acid capable of holding C_1 on a level of oxidation of either formate or formaldehyde. Arrows are meant to indicate not single reactions, but processes which may involve several steps

would appear then that, if exchange of D occurred in a derivative of folic acid, two types of folic acid coenzyme may be envisioned, a "formyl" and a "hydroxymethyl" intermediate. The interrelationships between them are formulated provisionally in Fig. 1. $R_1(R_2)N$— in this scheme represents a biologically active form of folic acid capable of holding a C_1 fragment on a level of oxidation of either formate or formaldehyde.

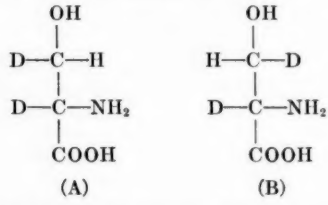
Since D is partly lost from $DC^{14}OONa$ and serine-β-$C^{14}D_2OH$ in the synthesis of purines, but not in the synthesis of serine and methyl groups, the reversal of Reaction 4 (Fig. 1) does not appear to be quantitatively important in the intact animal under our experimental conditions. Although Reaction 3 should be reversible, the stability of D in the conversion of the β-carbon of serine to methyl groups would not be appreciably affected by this equilibrium if serine is the major source of newly formed methyl groups (47) and Reaction 3 is not faster than Reactions 4 and 5. The postulation of an active C_1 intermediate capable of existence on a

level of oxidation of either aldehyde or acyl is analogous to the occurrence of a C_2 intermediate as active acetaldehyde and active acetyl (48).

Evidence for a "hydroxymethyl" intermediate in the metabolism of serine has been obtained recently in the conversion of the methyl group of sarcosine to the β-carbon of serine (49), in the synthesis of serine from glycine and formaldehyde, and in the exchange reaction $CH_2OH \cdot CHNH_2 \cdot COOH + C^+H_2NH_2 \cdot C^*OOH \rightleftharpoons CH_2OH \cdot C^+HNH_2 \cdot C^*OOH + CH_2NH_2 \cdot COOH$ (50, 51). In the latter two reactions tetrahydrofolic acid had a marked stimulating effect.

Possible Effects of Selective Removal of Protium or Deuterium—The conclusions presented here are valid only if selective cleavage of carbon-protium as opposed to carbon-deuterium bonds did not occur. Selection could take place in molecules of serine containing only 1 atom of D attached to the β-carbon. It could be due to either steric or kinetic effects.

The synthesis of Serine I, by addition of D_2 to ethyl formyl hippurate, $HC(OH) = C(NHCOPh) \cdot COOEt$, was such that none of the molecules contained more than 1 atom of D in the β-carbon and only one of the two stereoisomers of L-serine-2,3-D might have been produced, (A) or (B).



Since the ratio of C^{14}:D in the methyl groups was unaltered, preferential removal of deuterium did not take place. On the other hand, oxidation of the β-carbon of serine to the level of formate, during methyl group synthesis, might have occurred by preferential removal of protium. This could have been due to the greater strength of C—D than of C—H bonds, or to the stereospecificity of the enzymes involved. Although isomers (A) and (B) would both be subject to enzymatic attack, it might be expected that an atom of D would be removed from (A), while an atom of H would be removed from (B), or vice versa. Similar considerations would apply if oxidation occurred after cleavage of the serine molecule.

Serine II was synthesized to test the possibility of steric selection. Deuterium was introduced in such a way that, if Serine I consisted of only one of the two stereoisomers, Serine II would consist of only the other. Although the amount of deuterium in Serine II was too low to produce a desirable level of isotope in the methyl groups, the results obtained with this material (Table II) tended to confirm the findings with Serine I and

to suggest that steric factors are not responsible for the unaltered C^{14}:D ratios.

Serine III was prepared to test both steric and kinetic effects. In this compound 1.41 atoms of D were attached to the β-carbon in a random manner, so that 50 per cent of the molecules contained 2 atoms of D, 40 per cent 1 atom each of D and H, and 10 per cent 2 atoms of H. Selective cleavage from whatever cause could apply only to the 40 per cent of the molecules containing 1 D and 1 H. Molecules containing 2 atoms of D would have to lose 1 if the conversion of serine to methyl groups proceeded by way of "formate" or its derivatives. If in this process preferential breaking of C—H bonds were involved to any considerable extent, the methyl groups derived from Serine III should show a higher C^{14}:D ratio than was observed with Serine I. Such was not the case. The ratio of C^{14} dilution to D dilution is fairly close to 1, irrespective of type of serine used. It is, therefore, unlikely that either steric or kinetic effects are responsible for the observed stability of deuterium in the synthesis of methyl groups from serine.

In our preliminary report (2) it was mentioned that the unaltered C^{14}:D ratios could also be explained by a specific transfer of H or D from the β-carbon of serine to the pyrazine ring of folic acid, followed by utilization of the *same* molecules of reduced coenzyme for the formation of a methyl group. Pyridine nucleotides, which are known to mediate direct hydrogen transfer from certain substrates (52), might act in a similar manner. It is not possible at this time to assess the importance of such highly specific transfer reactions in the intact animal.

Relative Utilization of Serine and Glycine for C_1 Fragments—In order to equate the variable dosage and activity of the several precursors investigated, the observed isotope dilutions were converted to coefficients of utilization[4] and compiled in Table III.

It may be observed that the decarboxylation of serine to ethanolamine is an extremely rapid or extensive process, the coefficient of utilization for visceral protein serine being approximately equal to that found for the phospholipide ethanolamine. The value for the ethanolamine moiety of choline is approximately one-fifth as large. Utilization of serine for methyl groups is less extensive and more variable than for the ethanolamine moiety

[4] The coefficient of utilization (53) is derived from the isotope dilution formula, $b = a[(x/y) - 1]$, where a = millimoles of labeled compound administered per 100 gm. per day, b = millimoles of material per 100 gm. per day elaborated by the organism and used to dilute a, x = isotope concentration of a, and y = isotope concentration of the compound isolated. The coefficient of utilization = $1000/b = 1000/a[(x/y) - 1]$. It is expressed as 1000 times the reciprocal of b so that it will show an increase with increasing utilization. As modified for use with isolated tissue constituents, a becomes the total rather than the daily dose.

of choline. If experiment Serine I-C (intraperitoneal injection of serine into suckling rats) is omitted, the average ratio of activities of ethanolamine to methyl groups in choline is 2. In experiment Serine I-C this ratio is 5. In choline which was isolated from the mixed lipides of both carcass[5] and internal organs several days after the administration of serine-3-C^{14}, a ratio of 11 was found (28). The age of the animals and the experimental conditions, therefore, have a marked effect on the relative incorporation of label into the methyl and ethanolamine moieties of choline.

TABLE III

Relative Utilization of C_1 Precursors for Constituents of Visceral Proteins, Nucleic Acids, and Phospholipides

Precursor*	Coefficient of utilization,† gm. per mmole						
	Serine β-carbon	Pentose nucleic acid guanine (C-2+C-8)	Thymine (methyl)	Choline			Ethanolamine
				Methyl	N	C-1	
Serine I-A	73	37	25	8.3	11.5	11.1	
" I-B	44	43	22	7.1	11.9	11.8	
" I-C		18	12	1.7	7.7	8.8	
" II	45			4.4		13.5	
" III	41	46	16	4.8		10.6	49 (C-1)
$DC^{14}OONa$	9.0	35	9	3.7		5.2	
Glycine-2-C^{14}, N^{15}	11.0‡	4.5‡	3	1.1	5.0	1.0‡	26 (N^{15})
Glycine-1-C^{14}, N^{15}				0	5.5	0	
L-Histidine-2-C^{14} (45)	12	22		2.6		2.1	

* See Table I for details.

† See foot-note 4. Some of the data required for these calculations were published earlier (6, 33). The activity of choline methyl used in the calculations was the average activity per methyl group.

‡ The coefficient of utilization for the α-carbon of serine was 35; for C-5 of guanine, 30; for N-7 of guanine, 27; for C-2 of choline, 3.7.

It may be observed that, except in Serine I-C, the ratio of the coefficient of utilization of thymine-CH_3 to choline-CH_3 is close to 3 for all precursors. The β-carbon of serine is incorporated nearly equally into proteins and C-2 + C-8 of pentose nucleic acid guanine, whereas "formyl" precursors, such as histidine (45) and formate, are much more effective precursors of purines than of protein serine. Compared to serine, the other compounds (glycine, histidine, and formate) are poor precursors of methyl groups.

[5] In experiment Serine 1-A choline was isolated from the carcass lipides and found to have an activity only 7 per cent that of the internal organ lipides. This is at variance with the behavior of administered choline-N^{15} (23) which was incorporated equally into carcass and internal organs.

Glycine, however, differs from histidine and formate, since it is also poorly utilized for C-2 + C-8 of purines. Although classed with formyl precursors in Fig. 1, it would appear that glycine α-carbon more readily enters into equilibrium with serine or $R_1(R_2)N—CH_2OH$ than with $R_1(R_2)N—CHO$ (Fig. 1; *cf.* (47)). Histidine and formate, however, are more closely related to the $R_1(R_2)N—CHO$ pathway.

SUMMARY

L-Serine-3-C^{14},3-D was prepared by three independent procedures. Serines I and II contained not more than 1 atom of D per molecule in the β position (—CDHOH), the deuterium in Serine I having a stereochemical configuration opposite to that in Serine II. Serine III contained 50 per cent —CD_2OH, 40 per cent —CDHOH of random configuration, and 10 per cent —CH_2OH.

The conversion of these species of labeled L-serine, of glycine-1-C^{14},N^{15} and glycine-2-C^{14},N^{15}, and of $DC^{14}OONa$ in the rat to choline and ethanolamine was investigated.

It was found that the β-C^{14}, β-D, and N^{15} of serine are extensively utilized as a unit for ethanolamine synthesis, presumably by way of decarboxylation of the amino acid or a derivative. Glycine and formate are converted to serine prior to incorporation into ethanolamine.

All three compounds are also precursors of the methyl groups of choline, serine being the most effective. In this process the C^{14}:D ratio of the β-carbon of serine remains unaltered. Since the configuration of the deuterium in the β position has no influence on the results, steric or kinetic (isotope) effects do not appear to be responsible for the findings. These results suggest that one of the functions of folic acid in the metabolism of serine may be the transfer, in both cleavage and synthetic reactions, of a C_1 fragment on the level of oxidation of formaldehyde.

Several other reactions of C_1 units are discussed, and a scheme to account for them is tentatively proposed.

We are indebted to Mr. I. Sucher for the mass spectrometric analyses of D and N^{15}.

BIBLIOGRAPHY

1. Weissbach, A., Elwyn, D., and Sprinson, D. B., *J. Am. Chem. Soc.*, **72,** 3316 (1950).
2. Elwyn, D., Weissbach, A., and Sprinson, D. B., *J. Am. Chem. Soc.*, **73,** 5509 (1951).
3. Sulzer, H., *Angew. Chem.*, **25,** 1268 (1912).
4. Fenton, H. J. H., *J. Chem. Soc.*, **65,** 899 (1894).
5. Elwyn, D., and Sprinson, D. B., *J. Biol. Chem.*, **184,** 465 (1950).
6. Elwyn, D., and Sprinson, D. B., *J. Biol. Chem.*, **207,** 459 (1954).
7. Erlenmeyer, E., and Stoop, F., *Ann. Chem.*, **337,** 236 (1904).

8. Clarke, H. T., Johnson, J. R., and Robinson, R., The chemistry of penicillin, Princeton, 515 (1949).
9. Price, V. E., Gilbert, J. B., and Greenstein, J. P., *J. Biol. Chem.*, **179,** 1169 (1949).
10. King, J. A., *J. Am. Chem. Soc.*, **69,** 2738 (1947).
11. Nystrom, R. F., Yanko, W. H., and Brown, W. G., *J. Am. Chem. Soc.*, **70,** 441 (1948).
12. Cox, J. D., Turner, H. S., and Warne, R. J., *J. Chem. Soc.*, 3167 (1950).
13. Kilmer, G. W., and du Vigneaud, V., *J. Biol. Chem.*, **154,** 247 (1944).
14. Anker, H. S., *J. Biol. Chem.*, **176,** 1333 (1948).
15. Bloch, K., *J. Biol. Chem.*, **179,** 1245 (1949).
16. Radin, N. S., Rittenberg, D., and Shemin D., *J. Biol. Chem.*, **184,** 745 (1950).
17. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127,** 301 (1939).
18. Sprinson, D. B., and Rittenberg, D., *J. Biol. Chem.*, **180,** 707 (1949).
19. Graff, J., and Rittenberg, D., *Anal. Chem.*, **24,** 878 (1952).
20. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **130,** 703 (1939).
21. Sprinson, D., and Coulon, A., *J. Biol. Chem.*, **207,** 585 (1954).
22. Artom, C., *J. Biol. Chem.*, **157,** 585 (1945).
23. Stetten, D., Jr., *J. Biol. Chem.*, **140,** 143 (1941).
24. Chargaff, E., *J. Biol. Chem.*, **142,** 491 (1942).
25. du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140,** 625 (1941).
26. Lintzel, W., and Fomin, S., *Biochem. Z.*, **238,** 438 (1931). Guth, B., *Monatsh. Chem.*, **45,** 636 (1924).
27. Johnson, T. B., and Daschavsky, P. G., *J. Biol. Chem.*, **62,** 725 (1924–25).
28. Arnstein, H. R. V., *Biochem. J.*, **48,** 27 (1951).
29. Stetten, D., Jr., *J. Biol. Chem.*, **144,** 501 (1942).
30. Carter, H. E., and Melville, D. B., *J. Biol. Chem.*, **133,** 109 (1940).
31. Sakami, W., *J. Biol. Chem.*, **176,** 995 (1948).
32. Shemin, D., *J. Biol. Chem.*, **162,** 297 (1946).
33. Elwyn, D., and Sprinson, D. B., *J. Biol. Chem.*, **207,** 467 (1954).
34. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.*, **173,** 69 (1948).
35. Totter, J. R., Volkin, E., and Carter, C. E., *J. Am. Chem. Soc.*, **73,** 1521 (1951).
36. Karlsson, J. L., and Barker, H. A., *J. Biol. Chem.*, **177,** 597 (1949).
37. Ressler, C., Rachele, J. R., and du Vigneaud, V., *J. Biol. Chem.*, **197,** 1 (1952).
38. Keller, E. B., Rachele, J. R., and du Vigneaud, V., *J. Biol. Chem.*, **177,** 733 (1949).
39. Elwyn, D., and Sprinson, D. B., *J. Biol. Chem.*, **184,** 475 (1950).
40. Plaut, G. W. E., Betheil, J. J., and Lardy, H. A., *J. Biol. Chem.*, **184,** 795 (1950).
41. Stekol, J. A., Anderson, E. I., Weiss, S., and Hsu, P. T., Abstracts, American Chemical Society, 124th meeting, Chicago, 1C, 27C, Sept. (1953).
42. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, **176,** 165 (1948).
43. Keresztesy, J. C., and Silverman, M., *J. Am. Chem. Soc.*, **73,** 5510 (1951).
44. Cosulich, D. B., Smith, J. M., Jr., and Broquist, H. P., *J. Am. Chem. Soc.*, **74,** 4215 (1952).
45. Sprinson, D. B., and Rittenberg, D., *J. Biol. Chem.*, **198,** 655 (1952).
46. Knox, W. E., and Mehler, A. H., *J. Biol. Chem.*, **187,** 419 (1950). Mehler, A. H., and Knox, W. E., *J. Biol. Chem.*, **187,** 431 (1950).
47. Arnstein, H. R. V., and Neuberger, A., *Biochem. J.*, **55,** 259 (1953).
48. Gunsalus, I. C., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, 545 (1954).
49. MacKenzie, C. G., Sallach, H. J., and Frisell, W. R., Abstracts, American Chemical Society, 124th meeting, Chicago, 33C, Sept. (1953).

XUM

50. Blakley, R. L., *Biochem. J.*, **56,** p. xvii (1954); *Nature*, **173,** 729 (1954).
51. Kisliuk, R. L., and Sakami, W., *J. Am. Chem. Soc.*, **76,** 1456 (1954).
52. Vennesland, B., and Westheimer, F. H., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, 357 (1954).
53. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **155,** 243 (1944); **159,** 45 (1945).

ENZYMATIC OXIDATION OF α-CHYMOTRYPSIN*

By HENRY N. WOOD and A. K. BALLS

(From the Department of Biochemistry, Purdue University, Lafayette, Indiana)

(Received for publication, September 17, 1954)

Previous studies concerning the oxidation of chymotrypsin have demonstrated that a modified but still active end-product may be isolated (1–3). A preliminary report from this laboratory (4) was presented on the oxidation of chymotrypsin by means of a horseradish enzyme preparation. In a coupled oxidation system based upon the technique of Keilin and Hartree (5), 1 mole of α-chymotrypsin consumed 1 mole of oxygen, and the crystalline end-product retained half of its original esterolytic activity. The present work reports observations that lead to the conclusion that one tryptophan group is destroyed by this oxidation. Recently published work by Jermyn and Thomas (6) and by Wiltshire (7) strongly supports these findings in that the presence of several oxidative enzymes in horseradish root has been shown, among them a tryptophan oxidase through whose action 1 mole of tryptophan consumes 1 mole of oxygen.

Materials and Methods

Crystalline chymotrypsinogen,[1] obtained as a moist filter cake, was thrice crystallized, activated to chymotrypsin, again crystallized three times, exhaustively dialyzed against dilute acetic acid, then against water, and finally lyophilized.

A horseradish preparation was made as follows: 10 pounds of root were cleaned and sliced into 3 mm. disks, placed under cold running tap water for 10 days, then ground in a meat grinder. The pulp was steeped in a solution of sodium bicarbonate, pH 7.1, and stored at 0° overnight. The suspension was pressed free of solids in a hydraulic press at 8000 pounds per sq. in. The expressed juice was clarified by filtration with filter aid and made 40 per cent saturated with respect to ammonium sulfate. The precipitate which formed on standing was discarded. The clear filtrate was made 90 per cent saturated with ammonium sulfate; the precipitate which formed on standing was filtered and dialyzed against tap water. Insoluble material was then centrifuged. This procedure yielded a clear dark brown

* Journal Paper No. 805 of the Purdue University Agricultural Experiment Station, Lafayette, Indiana.

[1] Chymotrypsinogen was obtained from the Worthington Biochemical Sales Company, Freehold, New Jersey.

XUM

solution. The solution was next heated to 85° for 1 minute and then cooled rapidly in an ice bath, in order to permit the regeneration of peroxidases as described by Schwimmer (8). The heat treatment was originally employed in the hope that a great many enzymes, among them catalase, would be permanently destroyed, while peroxidase could be regenerated. This objective was only partially attained. Catalase was indeed greatly reduced, but never entirely removed, even by more drastic heating (100° for 5 minutes). Moreover a test of the enzyme preparation by the procedure of Durrum (9) showed the presence of four electrophoretically distinguishable substances, all of which were capable of oxidizing guaiacol in the presence of hydrogen peroxide.

Glucose dehydrogenase[2] was dissolved in distilled water, centrifuged to remove the solids, dialyzed, and lyophilized. A quantity equivalent to 0.1 mg. of this solid was used in all Warburg assays. The preparation used 0.24 ml. of O_2 per mg. per minute when tested in air at pH 5.6 with an excess of glucose.

A suspension of crystalline catalase[3] was diluted 250-fold.

Warburg Assay—The reaction system contained 25 mg. of α-chymotrypsin, 0.05 ml. of glucose dehydrogenase solution, 0.15 ml. of a 1 per cent solution of glucose in water, 2 γ (in 0.02 ml. of water) of protocatechuic acid, 0.2 ml. of catalase solution, 1.5 ml. of 0.066 M phosphate buffer (pH 6.4), and 0.25 ml. of horseradish preparation. Potassium hydroxide (0.2 ml. of a 10 per cent solution on filter paper) was used in the well. The reaction temperature was 30°.

Dialysis Sac Technique—The reaction system contained 500 mg. of α-chymotrypsin dissolved in 5 ml. of 0.066 M phosphate buffer (pH 6.4), 2.5 ml. of horseradish preparation, and 8 γ of protocatechuic acid, in a closed cellophane sac. The sac was immersed in 250 ml. of 0.00125 M H_2O_2, placed in a glass-stoppered tube protected from light. Blank tubes contained the same system without the chymotrypsin and without chymotrypsin and horseradish enzyme respectively. The tubes were gently agitated, and the decrease in hydrogen peroxide was measured colorimetrically by reaction with ammonium thiocyanate and ferrous ammonium sulfate in methanol (10). At the end of the experiment the protein was precipitated by ammonium sulfate at two-thirds saturation, and reprecipitated at pH 4.0 in the usual way for the preparation of α-chymotrypsin. A crystalline protein was thus obtained, which was recrystallized once. The crystals were indistinguishable on inspection from those of α-chymotrypsin. The preparation was dialyzed and lyophilized as usual. About 45 per cent of the original chymotrypsin was recovered at the final step.

[2] Glucose dehydrogenase was a commercial preparation, presumably made from *Aspergillus oryzae*.

[3] Catalase crystals obtained from the Worthington Biochemical Sales Company.

Phosphorylation—Part of the material obtained by the dialysis sac technique was phosphorylated with diphenyl chlorophosphate, and the product thereafter crystallized once from an ammonium sulfate solution at pH 4, as outlined by Jansen *et al.* (11). The crystals were needles resembling those previously described (12) for the corresponding derivative of α-chymotrypsin. They were dialyzed and dried in the frozen state as usual. The phosphorus content was determined by the method of Fiske and Subbarow after a micro-Kjeldahl digestion (13).

Analyses for Amino Acids—Histidine was determined microbiologically (14), tyrosine by the method of Herriott (15), and tryptophan by the method of Horn and Jones (16). Both acidic and basic protein hydrolysates were prepared by the technique of Levy and Chung (17). The esterolytic activity of chymotrypsin was measured against tyrosine ethyl ester by the method of continuous titration at pH 6.25 (18).

EXPERIMENTAL[4]

As shown in Fig. 1, the Warburg technique indicated that 1 mole of oxygen was consumed by 1 mole of chymotrypsin in this system.[5] The reaction came to a virtual standstill in 3 hours or less.

The oxygen consumption observed in the complete system except for chymotrypsin (lower curve, Fig. 1) was found to be practically the same as that obtained with glucose dehydrogenase, glucose, and catalase only. The same lower uptake was also observed when the peroxidase preparation was omitted but chymotrypsin was present. The data are reported in Table I. It follows therefore that both peroxidase and chymotrypsin are necessary for the additional uptake of oxygen, whereas catalase had no effect in this connection. The deliberate addition of catalase was probably unnecessary, as considerable catalase was present in the glucose dehydrogenase preparation. The only rôle of catalase in this system appears to be to decompose the hydrogen peroxide formed by glucose dehydrogenase but not used up in the oxidation of the chymotrypsin.

Fig. 2 describes the course of the reaction in the dialysis sac. The measurement of hydrogen peroxide in this system is not an accurate process, partly because of the instability of dilute solutions, but mainly because the horseradish enzyme still contained traces of catalase. Nevertheless the results approximate those by the Warburg technique, and the method

[4] The authors are greatly indebted to Dr. E. D. Schall of this Department for much assistance in the analyses of tryptophan; to Mr. H. Matsumoto of this Department for the microbiological assays of histidine; and to Mr. M. K. Walden of the Western Regional Research Laboratory, United States Department of Agriculture, for the peroxidase prepared according to Willstätter and Stoll.

[5] While the results reported here were all obtained in the presence of a catalytic amount of protocatechuic acid, several experiments in which this substance was omitted gave essentially the same results.

affords a means for preparing larger samples of oxidized chymotrypsin in the absence of the glucose dehydrogenase. Table II presents the results of three of these experiments, indicating the lower esterolytic activity of the

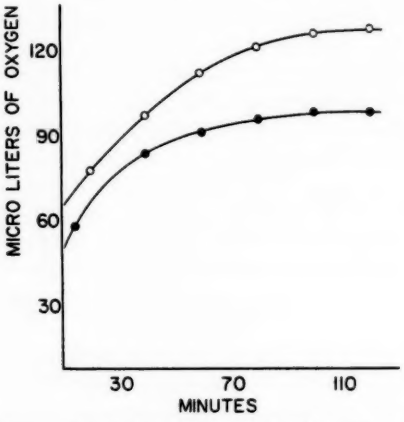


FIG. 1. Oxygen consumption (microliters, standard conditions) in the coupled oxidation of α-chymotrypsin by the peroxidase-glucose dehydrogenase system. ○, system containing 25 mg. of α-chymotrypsin; ●, same system without chymotrypsin. An additional uptake of 22.4 μl. thus represents the consumption of 1 mole of O_2 by 1 mole (taken as 25,000 gm.) of α-chymotrypsin.

TABLE I

Oxygen Consumed by 25 Mg. of α-Chymotrypsin in Presence of Peroxidase-Glucose Dehydrogenase System

Reactants	O_2 consumed	Per cent of original activity recovered
	μl.	
Complete system	123	56
No chymotrypsin	103	
" peroxidase	98	95
" chymotrypsin; no peroxidase	101	
Chymotrypsin only (in buffer)	−4	(100)

reaction product. (Only the protein from the largest run was crystallized. In the other runs the total precipitable protein was dialyzed, dried, and assayed.)

The crystalline protein isolated after oxidation in the dialysis sac (as described above) showed no uptake of oxygen when used in place of original chymotrypsin in the Warburg apparatus. It may therefore be concluded

that the oxidized protein was not merely a mixture of completely active and completely inactivated chymotrypsin.

Several analyses of the phosphorylated oxidized protein, which was totally inactivated, yielded 0.11 to 0.12 per cent P. This is practically

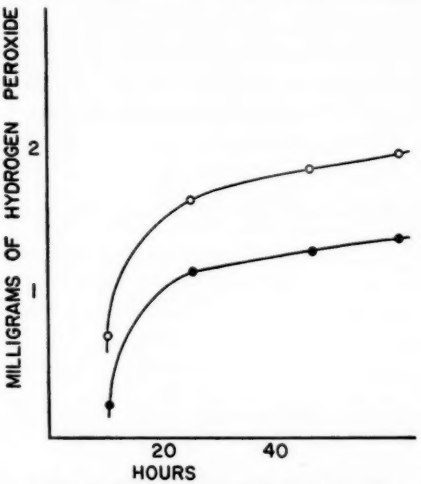


FIG. 2. Hydrogen peroxide consumption during oxidation in a dialysis sac. ○, system with 500 mg. of chymotrypsin; ●, system without chymotrypsin. All values are corrected for the (spontaneous) loss in hydrogen peroxide that occurred in a blank tube containing neither chymotrypsin nor peroxidase.

TABLE II

Specific Activity on Tyrosine Ethyl Ester

The activity is expressed in milliequivalents per mg. of enzyme protein per minute.

	Experiment A	Experiment B	Experiment C
Chymotrypsin used, *gm.*	1.5	0.25	0.5
Original activity, *m.eq.*	0.052	0.051	0.058
Oxidized protein (not isolated), *m.eq.*		0.0265	0.033
" " (crystals), *m.eq.*	0.0266		

the same as the published value of 0.127 per cent P for the phosphorylation product of the original α-protein (18). It is thus evident that this oxidation did not alter the amount of the group in chymotrypsin that undergoes phosphorylation, a result which is in contrast to those reported (3) for chymotrypsin oxidized in light.

Table III contrasts the results of the analyses of oxidized and original chymotrypsin for histidine, tyrosine, and tryptophan. The data indicate that substantially 1 mole of tryptophan was oxidized and that histidine and tyrosine remained unchanged. It is of interest that the horseradish-glucose dehydrogenase system was able to oxidize free tryptophan and also tyrosine, but not histidine.[6] Moreover, preliminary work indicated that chymotrypsinogen was also oxidized, and the breakdown as judged from oxygen uptake was more extensive than with chymotrypsin. The end-product in the case of chymotrypsinogen was (at least in good part) convertible to active enzyme by trypsin.

TABLE III

Analysis of Original and Oxidized α-Chymotrypsin

Amino acid	Moles per 25,000 gm. enzyme protein	
	Oxidized	Original
Histidine	1.6	1.6
Tyrosine*	11.5	11.2
Tryptophan†	6.1	7.3

* The tyrosine value, determined by the method of Herriott (15), is not corrected for cysteine.

† The protein was prepared for the assay of tryptophan by alkaline hydrolysis; all other determinations were made with acid hydrolysates (17).

DISCUSSION

It may be noted that hydrogen peroxide is definitely required for the enzymatic oxidation of chymotrypsin as described here. This fact classified the catalyst as a peroxidase by definition, but beyond that there is no information as to its precise nature or even its unity. Furthermore, there is no evidence that horseradish preparations made in other ways necessarily behave in the same manner. The results of Sizer (2) indicate that they do not. Moreover, a peroxidase preparation made by the method of Willstätter and Stoll (20) produced no oxygen uptake with chymotrypsin when tested by the method employed here.

It is interesting to contrast the effects of peroxidase on chymotrypsin with those of light as reported by Weil *et al.* (3). In the former case, the reaction reached a practical standstill with the removal of one tryptophan

[6] Spectroscopic analysis of tyrosine and tryptophan by the method of Goodwin (19) indicated a very high figure for tyrosine and tryptophan in oxidized chymotrypsin as compared to native chymotrypsin. Subsequent chemical methods, as indicated, resulted in the findings reported. Apparently this oxidized chymotrypsin contains a chromophore in the absorption region of the aromatic amino acids.

group, an uptake of about 1 mole of O_2 per mole of protein, and the formation of an apparently definite end-product having roughly half the esterolytic activity of the original. Histidine and tyrosine were not appreciably altered from their original values; neither was the phosphorus uptake on phosphorylation. In the latter case total inactivation was obtained after an uptake of 4 moles of O_2 per mole of protein, accompanied by a loss of one histidine and three tryptophan groups. The inactive protein could no longer be phosphorylated. Moreover, when half the activity had disappeared, half as much histidine had been destroyed as before and almost half as much tryptophan (1.1 moles per mole of protein).

It now appears, however, that chymotrypsin may be half inactivated by peroxidase without losing any histidine at all. On the other hand, the quantity of tryptophan lost is very nearly the same as that missing from the half active enzyme after photooxidation. It might be argued that these results taken together make tryptophan just as logical a suspect for the site of activity as histidine. To do so, however, would place undue weight on inactivation that is definitely only partial and evidently not connected with the phosphorus acceptor that is an admitted necessity for chymotryptic activity. Obviously the lost tryptophan is not a necessity for the esterolysis, but its presence in the protein does speed up that reaction.

With our present information it seems necessary to proceed with the viewpoint previously expressed (1) that, while the phosphorus acceptor (whatever it is) is a *sine qua non* in the active enzyme, other groups exist that are auxiliary thereto and required only for the fullest activity. A tryptophan group now appears to be one of these.

SUMMARY

α-Chymotrypsin was oxidized by a partly purified peroxidase from horseradish in the presence of hydrogen peroxide or a glucose dehydrogenase system to produce it. 1 mole of oxygen was consumed per mole of enzyme. The oxidized protein could be crystallized. It retained half of its original esterolytic activity when tested with tyrosine ethyl ester. Analysis indicated a loss of 1 mole of tryptophan, while histidine and tyrosine remained unchanged. The oxidized protein could be phosphorylated with diphenyl chlorophosphate to the same extent as the original protein. The results are contrasted with those of the photooxidation of chymotrypsin, in which histidine and tryptophan are both lost along with the ability to accept phosphorus. Thus the tryptophan removed by oxidation appears to function as an auxiliary rather than a necessary group in the action of the enzyme.

BIBLIOGRAPHY

1. Jansen, E. F., Curl, A. L., and Balls, A. K., *J. Biol. Chem.*, **189,** 671 (1951).
2. Sizer, I. W., *Advances in Enzymol.*, **14,** 129 (1953).
3. Weil, L. S., James, S., and Buchert, A. R., *Arch. Biochem. and Biophys.*, **46,** 266 (1953).
4. Wood, H. N., and Balls, A. K., *Federation Proc.*, **13,** 324 (1954).
5. Keilin, D., and Hartree, E. F., *Biochem. J.*, **39,** 293 (1945).
6. Jermyn, M. A., and Thomas, R., *Biochem. J.*, **56,** 631 (1954).
7. Wiltshire, G. H., *Biochem. J.*, **55,** 408 (1953).
8. Schwimmer, S., *J. Biol. Chem.*, **154,** 487 (1944).
9. Durrum, E. L., *J. Am. Chem. Soc.*, **73,** 4875 (1951).
10. Young, C. A., Vogt, R. R., and Nieuwland, J. A., *Ind. and Eng. Chem., Anal. Ed.*, **8,** 198 (1936).
11. Jansen, E. F., Nutting, M.-D. F., Jang, R., and Balls, A. K., *J. Biol. Chem.*, **179,** 189 (1949).
12. Jansen, E. F., Curl, A. L., and Balls, A. K., *J. Biol. Chem.*, **190,** 557 (1951).
13. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
14. Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Biol. Chem.*, **177,** 533 (1949).
15. Northrop, J. H., Kunitz, M., and Herriott, R. M., Crystalline enzymes, New York, 2nd edition, 309 (1948).
16. Horn, M. J., and Jones, D. B., *J. Biol. Chem.*, **157,** 153 (1945).
17. Levy, A. L., and Chung, D., *Anal. Chem.*, **25,** 396 (1953).
18. Balls, A. K., and Jansen, E. F., *Advances in Enzymol.*, **13,** 321 (1952).
19. Goodwin, T. W., and Morton, R. A., *Biochem. J.*, **40,** 628 (1946).
20. Willstätter, R., and Stoll, A., *Ann. Chem.*, **416,** 21 (1918).

AMMONIA AS AN INTERMEDIATE IN NITRATE REDUCTION BY BACILLUS SUBTILIS*

By LEO M. HALL and ROBERT MacVICAR

(From the Department of Agricultural Research, Oklahoma Agricultural Experiment Station, Stillwater, Oklahoma)

(Received for publication, July 26, 1954)

Despite great theoretical interest and practical importance, the biochemical pathway of nitrate reduction remains obscure. Considerable controversy exists, moreover, concerning the rôle of various probable intermediates in the reductive process. Indirect evidence that ammonia is an intermediate in the reduction of nitrate has been presented by Burris and Wilson (1) for *Azotobacter* and by Marshall *et al.* (2) for *Pseudomonas fluorescens* and *Pseudomonas denitrificans*. Direct evidence that ammonia is involved in N_2 fixation by the anaerobe *Clostridium pasteurianum* (Zelitch *et al.* (3)) provides additional support for viewing ammonia as an intermediate in the assimilation of nitrate. This communication presents direct evidence for the involvement of ammonia in the reduction of nitrate by a strain of *Bacillus subtilis*.

EXPERIMENTAL

Culture—The organism employed in this study was a strain of *B. subtilis* obtained from the Department of Bacteriology, Oklahoma Agricultural and Mechanical College. The organism was adapted to nitrate by subculture on medium of the following composition per liter: KH_2PO_4 3.96 gm., $NaNO_3$ 1.0 gm., glucose 20.0 gm., $MgSO_4 \cdot 7H_2O$ 0.04 gm., $FeSO_4$ 0.02 gm., NaCl 0.02 gm., $MnSO_4$ 0.08 gm., and biotin 0.1 mg. The pH was adjusted to 6.8 with KOH. Stock cultures were maintained on agar slants of the above medium, and inocula were prepared essentially as described by Sacks and Barker (4).

Incubation Techniques—Incubation experiments were carried out in 2 liter flasks, fitted with a fritted glass gas dispersion tube, in a water bath maintained at $37° \pm 1°$. Compressed air was passed through sterile filters and into the medium at a rate sufficient to maintain a constant dispersal of minute bubbles. The composition of the medium with respect to NO_3^- was followed from the time of inoculation until virtual depletion, at which time a sample was taken for analysis. Additional nitrogen as $KN^{15}O_3$ and

* Published with the approval of the Director, Oklahoma Agricultural Experiment Station. Supported, in part, by a contract with the Atomic Energy Commission, Division of Biology and Medicine.

$(NH_4)_2SO_4$ was then added to the culture. Samples of the medium and cells were removed serially until analysis again showed virtual exhaustion of nitrate or ammonia. Samples were transferred to flasks containing 2 ml. of hot 0.8 N H_2SO_4; the mixtures were heated to boiling, stoppered, and stored under refrigeration until analyzed.

Analytical Methods—Cells and culture medium were separated by centrifugation, and the supernatant liquid was carefully decanted. The cells were washed three times with distilled water and the washings added to the initial culture solution. The culture medium and washings were then made to volume for analysis. The residue of packed cells was transferred to semimicro-Kjeldahl flasks, and the mixture was rendered distinctly basic with NaOH and boiled for 5 minutes to remove occluded free ammonia. Total medium nitrogen was determined by a semimicromodification of the iron reduction procedure developed by Pucher *et al.* (5). Ammonia was determined by nesslerization following basic distillation of suitable aliquots of the diluted supernatant liquid plus washings or by the method of Pucher *et al.* (6). Nitrate was determined by a modification (2) of the method of Gad (7). Analysis for free hydroxylamine and oximino nitrogen was performed according to Endres' modification as given by Novak and Wilson (8). Paper chromatograms were prepared by the method of Berry *et al.* (9). The developing solvents were buffered phenol-water and lutidine-water. Both one- and two-dimensional chromatograms were made. Samples were prepared for N^{15} analysis essentially as suggested by Rittenberg *et al.* (10). N^{15} analyses were made on a mass spectrometer of the Nier type (Westinghouse) in the Mass Spectrometry Laboratory of the Texas Agricultural and Mechanical Research Foundation.

Results

Previous investigations in this laboratory with *Pseudomonas* species (2) had provided indirect evidence that ammonia was an intermediate in the reduction of nitrate to cellular nitrogen. To extend the investigation to other species, a nitrogen balance study of *B. subtilis* was undertaken. Typical results of such studies are presented graphically in Fig. 1. In this experiment, upon the exhaustion of the initial supply of NO_3-N, 25 mg. of N as $KN^{15}O_3$ containing 36.5 atom per cent N^{15} excess and 25 mg. of N as $(NH_4)_2SO_4$ were added simultaneously to the rapidly growing culture. Aliquots were withdrawn as described previously and analyzed for NO_3^-, NH_4^+, cellular nitrogen, and total nitrogen of the medium.

Inspection of Fig. 1 reveals that striking changes occurred during the 300 minute incubation period. Almost complete and extremely rapid disappearance of added nitrate occurred within the first 45 minutes of incubation. An abrupt increase in the ammonium nitrogen fraction took place

within the same period of time. Isotope data showed that the ammonia obtained by basic distillation of a sample of the medium taken at 45 minutes contained 12.85 atom per cent N^{15} excess. This high isotope concentration afforded direct evidence that ammonia, or some compound readily decomposing to yield ammonia, was an intermediate in the reduction of nitrate to cellular constituents. An inspection of the curves for ammonia and nitrate, as well as the curve for the medium N^{15}-N prior to 30 minutes,

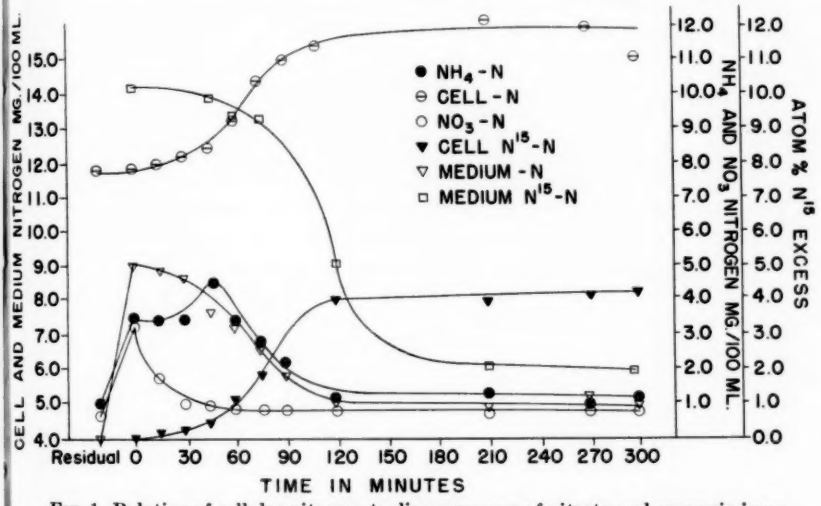

CELL AND MEDIUM NITROGEN MG./100 ML.
NH4 AND NO3 NITROGEN MG./100 ML.
ATOM % N15 EXCESS
NH4 - N
CELL - N
NO3 - N
CELL N15 - N
MEDIUM - N
MEDIUM N15 - N
Residual 0 30 60 90 120 150 180 210 240 270 300
TIME IN MINUTES

FIG. 1. Relation of cellular nitrogen to disappearance of nitrate and ammonia in an aerated culture of *B. subtilis*. 2.5 mg. of $N^{15}O_3$-N and 2.5 mg. of NH_4-N per 100 ml. added at zero time.

suggested that nitrate may be first converted to some other soluble nitrogenous compound which was then further reduced to ammonia.

Additional studies were also made with the view of determining the nature of possible intermediary compounds. The following analyses were performed in these studies.

Upon exhaustion of the original nitrate, 50 mg. of $KN^{15}O_3$-N and 50 mg. of $(NH_4)_2SO_4$-N were added to the culture. Aliquots of 25 ml. were withdrawn from the culture for the residual and initial samples. Thereafter, for the following 75 minutes, 100 ml. samples were withdrawn. Subsequently, 25 ml. samples were taken. To avoid decomposing any heat-labile substances, the samples were kept frozen until the analyses were completed. The analysis for ammonia was carried out according to the method of Pucher *et al.* (5), since it seemed possible that strongly basic

conditions in the previous experiment had decomposed some unstable intermediate to yield ammonia.

Ammonia nitrogen was also determined by the same method, after autoclaving the samples of medium (pH 2 to 3) for 1 hour at 18 pounds pressure. The results were very similar in both instances, indicating that hydrolysis under conditions known to effect decomposition of most amides did not cause liberation of ammonia.

Nitrate nitrogen was determined both colorimetrically and by reduction with Devarda's alloy after removal of ammonia. Similar values were

TABLE I

Changes in NO_3^--N and NH_4^+-N Content of Medium of Rapidly Growing Culture of B. subtilis

5.0 mg. of $KN^{15}O_3$-N and 5.0 mg. of $(NH_4)_2SO_4$-N per 100 ml. added at zero time.

Time	Nitrate nitrogen	Ammonium nitrogen
min.	*mg. per 100 ml.*	*mg. per 100 ml.*
Residual	0.3	1.2
0	5.3	6.1
10	5.1	6.3
20	4.7	6.7
30	4.2	6.5
45	3.7	8.0
60	2.9	7.0
75	1.9	5.3
105	1.0	3.7
135	0.3	1.7

obtained by both methods, indicating that alkaline reduction did not convert any intermediate to free ammonia.

The results of analysis for ammonia and nitrate nitrogen are presented in Table I. Examination of these data shows that the results were very similar to those obtained when less refined analytical procedures were employed (Fig. 1). The rate of nitrate disappearance was not as rapid as in the former experiment, but a proportionately larger increase in the ammonia concentration was evident, being nearly double that obtained previously. This greater concentration increased the sensitivity with which changes in ammonia N content could be followed.

The medium was also analyzed for nitrate, free hydroxylamine, and oximino nitrogen. No indication of these forms of nitrogen was obtained. According to Csaky (11), hyponitrous acid and nitrohydroxamic acid also give the typical color reaction with these reagents.

Paper chromatographic examination of the medium for α-amino nitro-

gen compounds at varying times throughout the incubation period revealed the presence of glutamic acid in detectable amounts. Other amino acids were present only in amounts below or approaching the sensitivity of the technique. The concentration of glutamic acid was fairly constant at less than 25 γ per ml. This low and uniform concentration of these compounds suggests that they are arising as the result of the breakdown of cellular constituents rather than from the reaction of an intermediate in the reduction of NO_3^- with a suitable carbon source.

DISCUSSION

The primary formation of nitrite from nitrate is, and on satisfactory grounds, generally considered to be the first step in the reduction of nitrate. The mechanism of further reduction of nitrite poses an interesting problem, since the possible intermediate compounds in a reduction chain from nitrite to amino configuration are limited. Despite this fact, no satisfactory evidence at present exists for the actual participation of such compounds as nitroxyl, its dimer hyponitrous acid, the hypothetical compound dioxyammonia, or hydroxamic acid. Since it is probable that the reduction and assimilation of nitrate represent a stepwise process, hydroxylamine might be assumed to be the precursor of ammonia. Hydroxylamine and ammonia may, however, be part of a mechanism in which the precise pathway of amino acid synthesis is dictated by the availability of suitable carbon compounds as acceptors. Amino acid formation appears possible either from hydroxylamine via the oxime or from ammonia via the α-imino acid; there is little reason to believe that the two mechanisms are mutually exclusive.

The evidence presented here does not support the view, however, that hydroxylamine constitutes the key intermediate in the reductive conversion of nitrate to cellular constituents. It should be noted, however, that the rates of formation and disappearance of hydroxylamine and related compounds may be such that their detection was not possible by the methods of analysis employed in these experiments. The analysis for hydroxylamine and oximino nitrogen was essentially that of Virtanen and Csaky (12). Consequently, it is significant that cultures of *B. subtilis*, in contrast to yeast, failed to show the presence of hydroxylamine or oximino N.

The results of these experiments do, however, show striking similarity to those obtained by Zelitch *et al.* (3) in which *Clostridium* fixes N_2. Since the pathways of nitrogen fixation by *Clostridium* and of nitrate assimilation by *B. subtilis* both involve ammonia as a common intermediate, it is likely that the mechanisms involved are similar and probably are identical in some respects. Strictly speaking, the highly specific data from these

studies, indicating ammonia to be an intermediate in biological nitrate reduction, should be restricted to *B. subtilis*. However, comparison of the biochemistry of biological nitrate reduction, particularly as indicated by N^{15} studies conducted in this laboratory and elsewhere, and the evidence for a common pathway for N_2 fixation by various biological agents suggest that NH_3 may be an intermediate common to many species, but not in such concentrations to permit ready detection.

SUMMARY

In vigorously aerated cultures of *Bacillus subtilis*, which had been adapted to nitrate nutrition by long serial subculture, extremely rapid disappearance of $N^{15}O_3^-$ occurred even when NH_4^+ was present. Analyses of components of the medium and use of tracer techniques both indicated that ammonia was an intermediate in the reductive pathway from NO_3^- to cellular N. Hydroxylamine, oximino nitrogen compounds, hydroxamic acids, or hyponitrous acid was not detected under the conditions and with the methods used.

The generous assistance of Mr. Glen D. Hallmark of the Mass Spectrometry Laboratory of the Texas Agricultural and Mechanical Research Foundation is acknowledged.

BIBLIOGRAPHY

1. Burris, R. H., and Wilson, P. W., *J. Bact.*, **52,** 505 (1946).
2. Marshall, R. O., Dishburger, H. J., MacVicar, R. W., and Hallmark, G. D., *J. Bact.*, **66,** 254 (1953).
3. Zelitch, I., Rosenblum, E. D., Burris, R. H., and Wilson, P. W., *J. Biol. Chem.*, **191,** 295 (1951).
4. Sacks, L. E., and Barker, H. A., *J. Bact.*, **58,** 11 (1949).
5. Pucher, G. W., Vickery, H. B., and Leavenworth, C. S., *Ind. and Eng. Chem., Anal. Ed.*, **7,** 152 (1935).
6. Pucher, G. W., Leavenworth, C. S., and Vickery, H. B., *Ind. and Eng. Chem., Anal. Ed.*, **7,** 191 (1935).
7. Gad, G., *Kleine Mitt. Mitglied. Vereins Wasser-, Boden- u. Lufthyg.*, **15,** 82 (1939); *Chem. Abstr.*, **34,** 5213 (1940).
8. Novak, R., and Wilson, P. W., *J. Bact.*, **55,** 517 (1948).
9. Berry, H. K., Sutton, H. E., Cain, L., and Berry, J. S., *Univ. Texas Pub., No. 5109,* 21 (1951).
10. Rittenberg, D., Keston, A. S., Rosebury, F., and Schoenheimer, R., *J. Biol. Chem.*, **127,** 291 (1939).
11. Csaky, T. Z., *Acta chem. Scand.*, **2,** 450 (1948).
12. Virtanen, A. I., and Csaky, T. Z., *Nature*, **161,** 814 (1948).

PURIFICATION OF THE ANTERIOR HYPOPHYSEAL THYROTROPIC HORMONE*

By I. GORDON FELS,† MIRIAM E. SIMPSON, AND HERBERT M. EVANS

(From the Institute of Experimental Biology, University of California, Berkeley, California)

(Received for publication, August 3, 1954)

Interest in the purification of thyrotropic hormone (TSH) has lagged behind the other seemingly more important hormones of the anterior pituitary, and progress in this field has lapsed proportionally. In 1940 Fraenkel-Conrat and coworkers (1) published a procedure by which a potent preparation could be obtained from beef pituitaries. An unusually detailed description of the hormonal contaminants also was given so that the preparation could be properly evaluated. In 1945 Ciereszko (2) reported the purification of TSH to the extent that the material had a minimal effective dose of 1 γ in the chick. A complete account of the hormonal contaminants, however, was not given. Recently (3) the present authors presented a procedure whereby a highly active preparation could be obtained which was free of other anterior lobe hormones at a total dose of 1 mg. in the hypophysectomized rat. The present communication is a modification and extension of that procedure and represents the results repeatedly obtained over a period of more than a year.

Methods

The hypophysectomized immature female rat (Long-Evans strain) has been used as the assay animal. The rats weighed about 70 gm. at the onset of the experiment and were 1 week postoperative. Throughout the experiment, they were maintained on an adequate diet.[1] Preparations to be tested were dissolved in distilled water and were injected intraperitoneally for 3 successive days, 0.5 ml. per day. The animals were sacrificed 24 hours after the last injection; the thyroids were removed, fixed, stained, and examined. The lowest concentration of the hormone in a series of dilutions which could effect a discernible histological response in each of three animals was considered the minimal effective dose. This response

* Aided by a grant (A-366) from the United States Public Health Service.

† Present address, Research Laboratory, Lucky Lager Brewing Company, San Francisco, California.

[1] Modified McCollum's Diet I consists of 67.5 per cent ground whole wheat, 15.0 per cent technical grade casein, 7.5 per cent skim milk powder, 0.75 per cent NaCl, 1.5 per cent Na_2CO_3, 6.7 per cent vegetable shortening, 1 per cent fish oil, KI solution (0.9 γ of iodine per gm. of diet).

was characterized by a slight increase in the height of the epithelium, some increase in cytoplasm, more vesicular, lighter staining nuclei, and the initiation of colloid resorption (1). The reliability of the method was based upon the ability to confirm, at least once, the minimal dose so assigned. At the termination of each experiment, completeness of the hypophysectomy was ascertained by examination of the pituitary site under a binocular microscope. The atrophy of the target organs as shown at autopsy, and on microscopic examination, gave confirmatory evidence of the completeness of the operation. Preliminary screening to remove incompletely hypophysectomized animals had already been accomplished in the preinjection period by analyzing the weight gain. No results have been included here unless completeness of hypophysectomy was ascertained in these ways.

The day-old, inbred white Leghorn male chick has been tested repeatedly as an assay animal but has not proved to be as dependable as the hypophysectomized rat. Whereas it was possible to note gravimetric changes in the thyroids with increasing dose levels, no such correlation could be found within a wide range of doses on histological examination of the thyroids. Furthermore, the variability of the gravimetric responses was considerable from week to week. These inadequacies of the chick test have been noted by other observers (2, 4).

The activity of the preparations was confirmed by the radioiodine uptake of the thyroid 2 weeks following hypophysectomy. The animals were maintained on the same diet as in previous tests, except that the iodide supplement was not included in the diet so that the total iodine content of the diet was 0.1 γ per gm. Three daily doses of TSH were given intraperitoneally, and, 4 hours after the last injection, 1 to 3 μc. of carrier-free KI^{131} was given intraperitoneally. The animals were sacrificed 24 hours after the radioiodine injection; the thyroids were removed and dissolved in 0.2 ml. of 2 N NaOH heated in a boiling water bath. The volume was brought to 10 ml., and 0.5 ml. aliquots were plated in triplicate and dried for radioactivity determination with the Geiger counter. The usual corrections for background and decay were made. The minimal effective dose was considered as the lowest concentration of the hormone giving iodine uptake values statistically different from those of the controls and agreeing with the value assigned from the histological test in the hypophysectomized rat.

Chemical Procedure

All operations were performed at 0–5°.

Step I—2 kilos of frozen anterior lobes of beef pituitaries were ground in an electrically driven mill to a mash by using $\frac{1}{8}$ and $\frac{1}{16}$ inch screens succes-

sively. The mash was extracted for 24 hours with 12 liters of cold distilled water at pH 11.5, with CaO slurry for the pH adjustment. The supernatant fluid was siphoned and the residue was reextracted with an additional 12 liters at pH 11.5 for 24 hours. The residue which was allowed to settle overnight was centrifuged and washed with 2 liters of water. The supernatant fluids and washings were combined, and the residue was discarded.

Step II—Gaseous CO_2 was bubbled through the solution at a slow rate until pH 8.7 was reached. The precipitate was allowed to settle overnight. The cherry-red supernatant fluid was separated by siphoning and the precipitate was discarded. Additional CO_2 was added to about pH 6, and a final pH 5.0 was obtained by the slow addition of cold 1 N HCl with efficient stirring, about 300 ml. of the acid usually being required. The precipitate was allowed to settle overnight. The clear red supernatant fluid was siphoned and the precipitate was discarded.

Step III—To the supernatant fluid solid ammonium sulfate was added slowly with mechanical stirring to a final concentration of 3.0 M. The precipitate was allowed to settle overnight and the supernatant fluid was discarded. The precipitate was dissolved in 4 liters of 1 per cent NaCl, and solid ammonium sulfate was added slowly to a final concentration of 0.6 M. The precipitate which formed overnight was discarded. Solid ammonium sulfate now was added to the supernatant fluid to bring the final concentration back to 3.0 M. The precipitate was allowed to settle overnight. The supernatant fluid was discarded, and the precipitate was again taken up in 4 liters of 1 per cent NaCl. Solid ammonium sulfate was added to 1.2 M. After settling overnight, the precipitate was removed, and additional ammonium sulfate was added to the supernatant fluid to a final concentration of 2.0 M. The precipitate was recovered the following day, dialyzed until free of salt, and lyophilized, with acetone and frozen CO_2 as the freezing mixture.

Step IV—Oxycellulose (10 to 12 per cent carboxyl) was prepared according to the procedure of Astwood *et al.* (5), with the exception that slightly more dilute acetic acid was used in the final step.

3.5 gm. of the dried 2 M precipitate were stirred overnight with 7.0 gm. of the prepared oxycellulose in 1.4 liters of 0.08 N acetic acid. The oxycellulose, after being removed by centrifugation, was discarded and the clear supernatant fluid, containing the major portion of the TSH, was lyophilized.

Step V—2 gm. of the material after adsorption were dissolved in 200 ml. of cold distilled water and cold 10 per cent trichloroacetic acid (TCA) was added slowly with mechanical stirring to a final concentration of 2.5 per cent TCA. The initial concentration of the TCA was determined by ti-

tration with NaOH. The addition of the acid should take about 20 to 30 minutes. The precipitate was removed and the supernatant fluid brought to 500 ml. with cold 2.5 per cent TCA. Cold saturated NaCl in 2.5 per cent TCA solution was added dropwise with stirring until a final concentration of 45.6 per cent NaCl-TCA (volume per volume) was obtained, that is, 420 ml. of the NaCl-TCA solution. This was equivalent to a final concentration of 15 per cent NaCl in 2.5 per cent TCA. The precipitate was centrifuged immediately, dialyzed free of salts, and lyophilized. To the supernatant fluid solid NaCl was added slowly to saturation. This required about 160 gm. of NaCl. The precipitate was dialyzed and lyophilized. The supernatant fluid was discarded. It is important that Step V proceed without interruption because of the sensitivity of the TSH towards acid. The yields and potencies of the various fractions are given in Table I. It is interesting to note that, regardless of the progressive purification, the Kjeldahl nitrogen values remained essentially the same, about 13 per cent. It suggests that the alkaline extraction was particularly effective in concentrating similar proteins of relatively low nitrogen content.

TABLE I

Yields and Potencies in Purification of TSH

Step No.		Yield per 2 kilos	Per cent nitrogen (Kjeldahl)	TSH, minimal effective dose
		gm.		γ
I	pH 11.5 extract	237.5	12.5	250–300
II	" 8.7 ppt.	190		5000
	" 5.0 "	24	13.3	5000
III	1st 3 M ppt.	18	13.7	100 *Ca.*
	1st 3 " supernatant fluid	28		5000
	0.6 M ppt.	5		3000 *Ca.*
	2nd 3 M ppt.	12	12.9	50–100
	1.2 M ppt.	3		100–200
	2 M ppt.	7	12.9	25
IV	2 " " postadsorption	5.2	12.9	25
V	1st NaCl-TCA ppt.	0.39	11.8	5
	Saturated NaCl-TCA ppt.	0.34	12.2	5

DISCUSSION

A departure from the original procedure (3) was the substitution of an alkaline extraction for the NaCl-acetic acid originally used. This change is based on the experience that the NaCl-acetic acid extracts are difficult to centrifuge. Furthermore, the TSH appears to be more stable in alkaline

solutions than in acids. The extraction with $Ca(OH)_2$ is similar to that used in the preparation of growth hormone (6).

Adsorption with oxycellulose in 0.08 N acetic acid is performed to eliminate the adrenocorticotropic hormone (ACTH) (5). Attention must be given to the concentrations of protein and adsorbent to obtain maximal recovery of TSH and at the same time adsorb the ACTH. The optimal concentration was found to be 1 part by weight of protein to 2 parts of adsorbent in a large volume of 0.08 N acetic acid. Under these conditions the recovery of protein was in the neighborhood of 75 per cent with no apparent loss of TSH potency, and no ACTH in the final preparation at a total dose of 3 mg. When less adsorbent was added, more protein was recovered, but ACTH was present invariably in the final preparations. In no instance has it been possible to recover TSH once it was adsorbed; elution with acetic acid, dilute HCl, or pyridine was equally unsuccessful.

TABLE II

Influence of Final TCA Concentration on TSH Recovery

Per cent final concentration TCA	Per cent protein yield	TSH, minimal effective dose
		γ
1	24	10
2	17	5
2.5	17	5
4	5	10
8	4	20

The previously reported fractionation of the 2 M material with acetone (3) has been abandoned in favor of the far more efficient precipitation with trichloroacetic acid. The fractionation with acetone was found difficult to control, as the time required for precipitation was unpredictable. Furthermore, prolonged standing in the organic solvent frequently resulted in reduced potency of the product. Both these points have been stressed by Fraenkel-Conrat *et al.* (1).

TCA has been used by other workers in the purification of TSH, although not always with success, especially when applied to crude preparations (2, 7). The applicability of TCA in later stages of purifications was suggested by the observation, previously reported (3), that TSH is soluble in high concentrations of TCA. It was applied in the present procedure to the 2 M material after the adsorption step, and the optimal concentration was found to be 2.0 to 2.5 per cent. The influence of the TCA concentration upon the recovery of TSH is shown in Table II.

Some TSH was lost in the TCA precipitate, and, by resolution in water and reprecipitation with TCA, additional TSH could be recovered. The

fraction was, however, contaminated with interstitial cell-stimulating hormone (ICSH) which was difficult to remove in the subsequent steps. For this reason, a second precipitation was not performed to increase the TSH recovery.

Any ICSH present in the initial TCA supernatant fluid was removed by fractionation with NaCl with 2.5 per cent TCA as solvent. Most of the ICSH was found in the 15 per cent NaCl precipitate, at which stage most of the TSH remained in the supernatant fluid and was precipitated by saturation with NaCl. No activity remained in the supernatant fluid after saturation. Large losses of TSH were incurred in this step, since considerable quantities of the TSH were precipitated with the ICSH at 15 per cent NaCl. In spite of this loss, as well as others, the final recovery of potency based on the original alkaline extract is about 6 per cent.

Biological Characterization

The final product as obtained by this procedure had a minimal effective dose of 5 γ as assayed in the hypophysectomized rat. The minimal effective dose was the same as that determined by the radioiodine uptake by the thyroid. A dose of 40 γ resulted in an I^{131} uptake equal to or greater than that of normal control rats of the same age (Table III).

Although these small dose levels were capable of influencing the thyroid of the hypophysectomized rat both histologically and functionally after the short period of treatment used, it is of interest that no increased thyroid weight resulted from injection of levels of TSH 100 times the minimal effective dose.

Thyrotropic hormone is known to be capable of synergizing the effects of growth hormone (8).[2] Presumably the enhancement of growth hormone activity is due to the discharged thyroxine (9). Quantitative experiments to be described elsewhere demonstrate that the present thyrotropic hormone, like thyroxine, augments the activity of growth hormone as measured by the tibia line test and the body weight test in hypophysectomized rats.

Contamination with Other Hormones

Lactogenic Hormone—The preparation has been tested in pigeons by the systemic test (10) and found to be free of lactogenic hormone at a total dose of 5 mg. By using the local crop test (11) which is reported to be able to detect 0.1 γ of lactogenic hormone, the preparation was found negative for lactogenic hormone at a total dose of 100 γ. From this it is assumed that lactogenic hormone contamination in the preparation is less than 0.1 per cent.

[2] The synergistic effect of TSH on growth hormone in the tibia test has been noted also by Dr. I. I. Geschwind of the Hormone Research Laboratory in a private communication.

Adrenocorticotropic Hormone—A test of the TSH preparation for contamination with ACTH by the adrenal repair test in hypophysectomized rats (12) showed that it was free of ACTH at a total dose of 3 mg. Assayed by the ascorbic acid depletion test (13), as modified by Geschwind *et al.* (14), 250 γ of the TSH preparation were found to have the same activity as 0.003 unit of U. S. P. ACTH per mg. Since the purest ACTH has a potency of about 150 units per mg., the contamination of the preparation by ACTH may be considered negligible.

Growth Hormone—The thyrotropic hormone gave no evidence of contamination with growth hormone as tested by the tibia line test in the hypophysectomized rat (15) at a total dose of 3 mg. In the body weight test (16) the thyrotropic preparation caused an average body weight increase in hypophysectomized rats of 7 gm. in 10 days when given at a total dose of 5 mg. This gain is less than the minimal gain required for significance.

TABLE III

Influence of TSH on Radioiodine Uptake by Hypophysectomized Rat Thyroid

Total dose TSH	Average thyroid weight	Average iodine uptake*
γ	*mg.*	*per cent*
40	6.4 (5)†	15.4 ± 3.1
10	6.6 (5)	5.4 ± 2.0
5	5.8 (5)	4.3 ± 0.9
Hypophysectomized controls	5.9 (5)	1.5 ± 0.3
Normal controls	7.6 (5)	10.2 ± 2.5

* The average deviations from the mean are indicated in the last column.

† The number of animals per group is indicated parenthetically.

Gonadotropic Hormones—The preparation has been found free of follicle-stimulating hormone at a total dose of 3 mg., as judged by initiation of follicle growth in the hypophysectomized rat (17). At the same total dose level there was usually no repair of interstitial cells (18). Occasionally the minimal effective dose for ICSH of a preparation was between 2 and 3 mg. Since purified ICSH preparations cause repair at 0.01 mg., these preparations of TSH were contaminated to the extent of 0.3 per cent with ICSH.

Intermedin—Preliminary assay of the preparation for intermedin in *Rana pipiens* (19) showed a strong positive reaction at 0.1 γ, and, since this assay is capable of detecting 0.001 γ of intermedin, the contamination is more than 1 per cent.

Table IV summarizes the biological characteristics of the present material in comparison with preparations of other workers. A strict comparison between potencies is not possible when different species (chick and rat) are used in the TSH assay.

TABLE IV

Comparison of TSH Preparations

Preparation	TSH, minimal effective dose	Assay, histological	ICSH	FSH	GH	ACTH	Lactogenic
	γ						
Fraenkel-Conrat *et al.* (1)	90–110*	Rat†	+ at 100 γ* (I. T. repair)	+ at 1 mg.*	+ at 1 mg.* (weight gain test)	− at 3 mg. (adrenal weight test)	− at 100 γ,* + at 400 γ* (local crop test)
Ciereszko (2)	1	Chick	Unknown	Unknown	− at 5 mg. (weight gain test)	Unknown	− at 5 mg. (systemic test)
U. S. P. reference standard	10*	Rat†	+ at 1 mg. (I. T. repair)	+ at 1 mg.	*Ca.* 2.5% GH standard (weight gain test)	0.02 i.u. per mg.	0.25 i.u. per mg.
Thyropar (Armour)	20*	" †	+ at 1 mg.* (I. T. repair)	− at 3 mg.*	− at 3 mg.* (tibia line test)	+ at 1 mg.* (adrenal repair)	Unknown
Fels *et al.*	5*	" †	− at 3 mg.*	− at 3 mg.*	− at 3 mg.* (tibia line test)	− at 3 mg.* (adrenal repair)	− at 5 mg.* (systemic test)
	5*	" † iodine uptake			− at 5 mg.* (weight gain test)	0.002 i.u. per mg. (ascorbic acid depletion)	− at 100 γ (local crop test)

ICSH = interstitial cell-stimulating hormone, FSH = follicle-stimulating hormone, GH = growth hormone, I. T. = interstitial tissue.

* Assay performed in this laboratory.

† Hypophysectomized.

Chemical and Physical Properties

The final preparation of TSH as obtained by this procedure was a white amorphous powder which was very soluble in distilled water and aqueous solutions of salts, alcohol, and acetone. The low Kjeldahl nitrogen, 12.2 per cent, is indicative of a conjugated protein, and this is supported by the positive Molisch test for carbohydrate. The solubility in trichloroacetic acid and dializibility through cellophane membranes suggest a substance of relatively low molecular weight. The diffusibility accounts for a 10 to 20 per cent unavoidable loss of material during the dialysis in the final step of preparation.

Several attempts have been made to ascertain the homogeneity of the preparation by the usual criteria of protein purity.

Electrophoresis—Electrophoretically, the preparation yields a fast moving, blurred boundary which cannot be measured in the pH range 4.0 to 9.2 with acetate, phosphate, Veronal, or borate buffers. The unsatisfactory electrophoretic pattern was undoubtedly due to the inability of the material to reach an osmotic equilibrium with the buffer, thus giving rise to a large conductivity difference across the boundary.

Filter paper electrophoresis, which requires no preliminary dialysis against the buffer, has been somewhat more successful. By the method of Kunkel and Tiselius (20), it has been possible to detect at least two components in alkaline buffers. In acidic buffers, the adsorption by the paper renders the procedure useless.

The experiments were conducted in Veronal and borate buffers, pH 8.0 to 9.2 and ionic strength 0.1. The current used was about 5 ma. for 48 hours. The measure of endoosmotic flow was obtained by the use of dextran which was lightly sprayed with alcoholic brom phenol blue in the area of its application. In order to follow the component possessing the activity, two superimposed papers (Whatman No. 20) were used. About 5 mg. of the material were placed on the papers, and, after the experiment, the upper paper was removed and cut into 1 cm. strips on both sides of the origin. The strips were eluted either with 2 ml. of dilute NH_4OH or Na_2CO_3, and aliquots were taken for protein determination by the Lowry method (21). The usual brom phenol blue procedure for locating the material (20) could not be used because most of the TSH was lost in the washing process. Once the components were located, the lower paper was cut and eluted for determination of biological activity.

Although an accurate determination of an isoelectric point was not possible by use of the two papers, it appears from the mobility of the active component that the isoelectric point is in the neighborhood of pH 9. Steelman *et al.* (22), using an electrical transport procedure, estimated the isoelectric point to be between pH 8.0 and 8.5.

XUM

Ultracentrifugation—1 per cent solutions of the final material in Veronal buffer, pH 8.42, and borate buffer, pH 9.20, ionic strength 0.1, gave a sharp symmetrical sedimenting boundary in both buffers when the synthetic boundary cell was used (23). The sedimentation constant obtained in Veronal was 1.02 and in borate 1.03. This would correspond to a molecular weight of 6000 to 10,000, making plausible assumptions for the frictional constant and partial specific volume. It is noteworthy that a sedimentation constant of 1.0 and a probable molecular weight of 10,000 were estimated by White (24) in 1944 at a time when sedimentation technique was not perfected for study of low molecular weight compounds.

Chromatography—The use in column chromatography of adsorbents such as silica, Permutit, oxycellulose, or the carboxylic resin IRC-50 resulted in irreversible adsorption of TSH, whereas with diatomaceous earth (Hyflo-Super-Cel) no adsorption occurred. Powdered cellulose (Solka-Floc) adsorbed the hormone from dilute acetic acid, and the elution could be accomplished quantitatively with dilute NH_4OH. This system was used as an analytical tool by making use of a pH gradient elution whereby the material after adsorption in dilute acetic acid was eluted by gradually raising the pH of the eluting agent. No elution was possible in acid pH. The use of gradient elution has been applied successfully in the analysis of complex carbohydrates by Alm (25). A general treatment of the method has been given by Alm, Williams, and Tiselius (26). Heideman (27) has used Solka-Floc without success for purification of TSH.

The procedure for use of Solka-Floc was as follows: About 25 gm. were washed with 0.2 N NH_4OH on a medium sintered glass filter with gentle suction until the filtrate did not absorb in the range 2700 to 2900 A. The adsorbent was then washed successively with 1 liter each of H_2O, 0.1 N HCl, H_2O, and finally 0.08 N acetic acid. The suspension in the latter solvent was then poured into a chromatographic tube measuring 37 × 0.9 cm. The adsorbent was allowed to settle for 24 hours. About 5 mg. of the purest TSH preparation dissolved in a minimal volume of 0.8 N acetic acid were added to the column. The walls were rinsed three times with 0.5 ml. of the same concentration of acid. The top of the column was attached to a reservoir or mixing chamber containing 25 ml. of 0.8 N acetic acid. A 50 ml. Erlenmeyer flask with an outlet at the bottom is suitable for this purpose.

A solution of 0.2 N NH_4OH was then added dropwise with magnetic stirring. The drop rate of the effluent was adjusted to 2 to 4 drops per minute. The entire apparatus was placed over an automatic fraction collector, and the process was followed by the Lowry method (21) for protein determination on a small aliquot. Judged from the color value, the total recovery was 92 per cent.

Fig. 1 illustrates the result of such a fractionation of the purified hormone on powdered cellulose. The demonstration of two main components is in harmony with the observations made with paper electrophoresis. Assay of the material from the two peaks showed activity only in Peak II. However, the activity was only about half the initial activity; thus inactivation presumably occurred in the column. The results shown here have been duplicated on preparations of TSH prepared at different times.

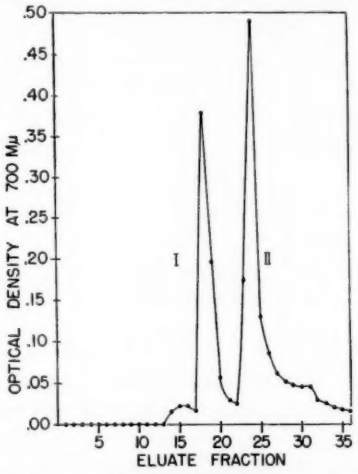


FIG. 1. pH gradient elution of purified TSH on powdered cellulose. 5 mg. of purified TSH dissolved in minimal volume of 0.08 N acetic acid were added to a powdered cellulose column, 37 × 0.9 cm. Reservoir of 25 ml. of 0.08 N acetic acid, connected to the column and magnetically stirred, received dropwise 0.2 N NH_4OH. Drop rate of effluent adjusted to 3 to 4 drops per minute; drop rate of NH_4OH to 3 times this rate. 1 ml. fractions collected. 0.1 ml. aliquot taken for determination. Peak I is inactive; Peak II possesses thyroid-stimulating activity.

By examination of the ultraviolet absorption spectra of material from the two peaks in dilute alkali it can be shown that Peak I is not an inactive product of Peak II. The molar tyrosine-tryptophan ratios of the two peaks were also different, being 1.3 for Peak I and 2.0 for Peak II. These values were obtained by the spectrophotometric method of Goodwin and Morton (28) with aliquots which possessed the same Lowry color value from each peak, and hence having the same concentration, provided the substances are closely related. A plot of wave-length *versus* optical density also showed the individuality of the materials, inasmuch as the curve for Peak I was considerably lower than that for Peak II.

The presence of ammonium acetate in the isolated Peak II made uncer-

tain any determination of protein concentration; hence an estimation of molecular weight based on tyrosine-tryptophan content could not be performed. The presence of carbohydrate in the biologically active Peak II was indicated by a positive Molisch test. Here again, the presence of ammonium acetate prevented a quantitative determination.

End-Group Analysis—The quantitative end-group analysis by the dinitrofluorobenzene method (29)[3] was applied to Peak II. The chromatogram of the hydrolyzed material after reaction with dinitrofluorobenzene indicated that no free amino end-group was present in the TSH molecule. This may be interpreted to mean that the end-group is proline, hydroxyproline, carbohydrate, or that the peptide chain is circular with no free amino groups.

SUMMARY

1. A procedure for the purification of beef anterior hypophyseal thyrotropic hormone is given.

2. The final product had a minimal effective dose of 5 γ, judged both by histological response and radioiodine uptake of the thyroid of the hypophysectomized rat.

3. The preparation was free of ACTH, growth hormone, interstitial cell-stimulating hormone, follicle-stimulating hormone as tested in hypophysectomized rats at a total dose of 3 mg., and free of lactogenic hormone at 5 mg. as tested in pigeons.

4. It yields a sharp, symmetrical sedimenting boundary in the ultracentrifuge, from which a sedimentation constant of 1.0 has been obtained.

5. The material was fractionated chromatographically into two components of which only one was active. The active component had a molar tyrosine-tryptophan ratio of 2.0, and contained carbohydrate; no free amino end-group was detectable.

The authors would like to express their indebtedness and appreciation to Dr. H. K. Schachman of the Biochemistry and Virus Laboratory for the ultracentrifugation experiments, to Dr. F. Carter of this laboratory for the lactogenic assays, to Dr. I. I. Geschwind of the Hormone Research Laboratory for the ascorbic acid depletion and intermedin assays, to Mr. David Chung of the Hormone Research Laboratory for the end-group analysis, and to Dr. S. Steelman of Armour and Company for a gift of Thyropar. The technical assistance of Miss H. Matsuzaki and Mr. J. Moss is gratefully acknowledged.

[3] Levy, A. L., and Chung, D., in preparation.

BIBLIOGRAPHY

1. Fraenkel-Conrat, J., Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, **135,** 199 (1940).
2. Ciereszko, L. S., *J. Biol. Chem.*, **160,** 585 (1945).
3. Fels, I. G., Simpson, M. E., Sverdrup, A., and Evans, H. M., *Endocrinology*, **51,** 349 (1952).
4. Bates, R. W., Riddle, O., and Lahr, E. L., *Endocrinology*, **29,** 492 (1941). Albert, A., Rawson, R. W., Merrill, P., Lennon, B., and Riddell, C., *J. Biol. Chem.*, **166,** 637 (1946).
5. Astwood, E. B., Raben, M. S., Payne, R. W., and Grady, A. B., *J. Am. Chem. Soc.*, **73,** 2969 (1951).
6. Fraenkel-Conrat, H., Meamber, D. L., Simpson, M. E., and Evans, H. M., *Endocrinology*, **27,** 605 (1940).
7. Loeser, A., *Klin. Wochschr.*, **11,** 1271 (1932). Jorgensen, M. N., and Wade, N. J., *Endocrinology*, **28,** 406 (1941). Albert, A., *Ann. New York Acad. Sc.*, **50,** 466 (1949).
8. Marx, W., Simpson, M. E., and Evans, H. M., *Proc. Soc. Exp. Biol. and Med.*, **49,** 594 (1942).
9. Smith, P. E., *Proc. Soc. Exp. Biol. and Med.*, **30,** 1252 (1933). Becks, H., Simpson, M. E., Evans, H. M., Ray, R. D., Li, C. H., and Asling, C. W., *Anat. Rec.*, **94,** 631 (1946). Evans, H. M., Simpson, M. E., and Pencharz, R. J., *Endocrinology*, **25,** 175 (1939).
10. Lyons, W. R., and Catchpole, H. R., *Proc. Soc. Exp. Biol. and Med.*, **31,** 305 (1933).
11. Lyons, W. R., *Proc. Soc. Exp. Biol. and Med.*, **35,** 645 (1937).
12. Simpson, M. E., Evans, H. M., and Li, C. H., *Endocrinology*, **33,** 261 (1943).
13. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, **42,** 379 (1948).
14. Geschwind, I. I., Williams, B. S., and Li, C. H., *Acta endocrinol.*, **8,** 247 (1951).
15. Greenspan, F. S., Li, C. H., Simpson, M. E., and Evans, H. M., *Endocrinology*, **45,** 455 (1949).
16. Marx, W., Simpson, M. E., and Evans, H. M., *Endocrinology*, **30,** 1 (1942).
17. Evans, H. M., Simpson, M. E., Tolksdorf, S., and Jensen, H., *Endocrinology*, **25,** 529 (1939).
18. Simpson, M. E., Li, C. H., and Evans, H. M., *Endocrinology*, **30,** 977 (1942).
19. Teague, R. S., Noojin, R. O., and Geiling, E. M. K., *J. Pharmacol. and Exp. Therap.*, **65,** 115 (1939).
20. Kunkel, H. G., and Tiselius, A., *J. Gen. Physiol.*, **35,** 89 (1951).
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193,** 265 (1951).
22. Steelman, S. L., Giffee, J. W., Jr., and Hawrylewicz, E. J., *Federation Proc.*, **11,** 292 (1952).
23. Schachman, H. K., and Harrington, W. F., *J. Polymer Sc.*, **12,** 379 (1954).
24. White, A., in Moulton, F. R., The chemistry and physiology of hormones, Washington, 1 (1944).
25. Alm, R. S., *Acta chem. Scand.*, **6,** 1186 (1952).
26. Alm, R. S., Williams, R. J. P., and Tiselius, A., *Acta chem. Scand.*, **6,** 826 (1952).
27. Heideman, M. L., Jr., *Endocrinology*, **53,** 640 (1953).
28. Goodwin, T. W., and Morton, R. A., *Biochem. J.*, **40,** 628 (1946).
29. Porter, R. R., and Sanger, F., *Biochem. J.*, **42,** 287 (1948).

XUM

THE FATE OF INJECTED CHOLESTENONE IN THE INTACT RAT*

By WILLIAM M. STOKES, WILLIAM A. FISH, AND FREDERICK C. HICKEY, O. P.

(From the Medical Research Laboratory, Providence College, Providence, Rhode Island)

(Received for publication, August 25, 1954)

Anker and Bloch (1) have shown that, when deuterium-labeled cholestenone (Δ^4-cholestene-3-one) is fed to rats, the sterol mixture from the carcass, blood, and liver becomes labeled, most of the deuterium being associated with cholestanol. However, a "small but significant" deuterium concentration was reported associated with cholesterol, after purification through the dibromide. While their observations exclude cholestenone as a major precursor of cholesterol, the possible conversion of a Δ^4-ketone to a 3β-hydroxy-Δ^5 compound is of interest. We have therefore determined the relative amounts of activity present in the purified cholestanol and cholesterol in rat liver after injection of cholestenone-4-C^{14} into the portal vein.

EXPERIMENTAL

The labeled cholestenone was prepared by published methods (2–4) and purified by chromatography on alumina. It melted at 82–83° and had a specific activity of 3.0×10^4 c.p.m. per mg.[1] The cholestenone was dispersed in saline (6) immediately before injection.

Male Wistar rats, weighing 300 to 370 gm., were used. Each was etherized and the duodenal loop delivered through an abdominal incision. After the portal vein was located, the solution containing cholestenone was slowly injected, the incision closed, and the rat allowed to recover. 1 hour after the injection the animal was sacrificed and the liver excised and hydrolyzed in ethanolic KOH. The non-saponifiable fraction was extracted with ether, and the sterols were isolated as digitonides.

To determine the possibility of contamination of the sterol digitonides with cholestenone (7), a mixture of 0.3 mg. of radiocholestenone and 5.0 mg. of cholesterol was precipitated. Less than 3 per cent of the cholestenone activity appeared with the digitonide.

* This work was supported by grants-in-aid from the American Cancer Society, upon the recommendation of the Committee on Growth of the National Research Council, and from the American Heart Association.

[1] All counts were made under comparable conditions with an Amperex G-60 tube and corrected to infinite thinness by the method of Gora and Hickey (5). Cholestenone was plated on planchets; sterol samples were counted as digitonides, filtered on fritted glass disks.

XUM

It was found that four-fifths of the activity in the liver non-saponifiable fraction was digitonin precipitable. The total digitonide fraction of one liver was cleaved with pyridine (8), carrier cholesterol added, and the mixture brominated. The crystalline dibromide was debrominated (9), a portion counted as the digitonide, and the remainder, with added carrier cholesterol, again brominated and debrominated. The specific activities of successive digitonides, corrected for carrier, were crude, 308; once regenerated, 96; twice regenerated, 25 c.p.m. per mg. Frantz and Bucher (10) report that two passages through the dibromide were required to reduce the cholestanol content of a cholesterol-cholestanol mixture from 5.0 to 0.15 per cent. Since repeated bromination does not effect complete separation of the above compounds, chromatography was used.

In the main experiment a 365 gm. rat was injected with 5.0 mg. of radiocholestenone in 1.0 ml. of saline. The liver weighed 10.6 gm., and its crude digitonide fraction, weighing 130.3 mg., had a specific activity of 608.3 c.p.m. per mg. and contained 53 per cent of the injected activity. 114.5 mg. of this crude digitonide were cleaved, and 29.7 mg. of carrier cholestanol[2] were added to the recovered sterol. This mixture was then converted to the I^{131}-labeled *p*-iodobenzoates and chromatographed by a method (11) which employs a collimated scintillation counter to follow the zones during development and to determine quantitatively the ester content of the developed zones. The developed cholestanol and cholesterol zones were symmetrical, overlapped slightly, and contained almost equal quantities of ester. The active length of the column was sectioned into twelve portions, each containing equal amounts of iodine activity. Each portion was eluted with ethanol-benzene, the esters were hydrolyzed (12), the sterols were extracted with ether, the ether was washed repeatedly with water to eliminate traces of potassium *p*-iodobenzoate-I^{131}, and the digitonides were precipitated and counted. The samples contained no detectable iodine activity, as evidenced by the fact that sample counts fell to background when a 346 mg. per sq. cm. aluminum absorber impervious to C^{14} β-rays, but essentially transparent to I^{131} γ-rays, was interposed. The results are recorded in Table I.

The cholestanol zone had a specific activity of 363 ± 6 c.p.m. per mg.; the cholesterol zone, 5.1 ± 2.1. While the specific activity was constant throughout the cholestanol zone, that of cholesterol went through a minimum. The higher activity of the lower end of this zone was probably due to slight overlapping with the cholestanol zone, while the specific activity of the top three fractions varied directly with the length of the column section taken. This variation can probably be attributed to contamination with free cholestanol derived from the slow hydrolysis of the cholestanyl

[2] Kindly provided by Professor Harold R. Nace of Brown University.

p-iodobenzoate on the column. Free sterols travel very slowly under the above chromatographic conditions.

60.9 mg. of cholesterol digitonide from Fractions 1 to 5 were cleaved with pyridine, the dried sterol was brominated in a centrifuge tube in 95 per cent ethanol (13), and the dibromide recrystallized twice from the same solvent. Three fractions were obtained: (1) the mother liquor of bromination and two ethanol washes, (2) the two mother liquors of recrystallization, and (3) the twice recrystallized cholesterol dibromide. The three fractions were dried under an air jet and *in vacuo*, debrominated (9), converted to the digitonides, and counted. Their specific activities were, respectively, 9.3, 10.0, and 0.8 c.p.m. per mg.

TABLE I

Chromatography of Rat Liver Sterols and Carrier Cholestanol after Injection of Labeled Cholestenone

Fraction No.	Column section, distance from top	Specific activity of digitonide	Component
	cm.	*c.p.m. per mg.*	
1	22.0–31.7	8.3	Cholesterol
2	31.7–33.9	3.6	
3	33.9–35.3	2.5	
4	35.3–36.6	4.5	
5	36.6–38.1	6.5	
6	38.1–41.0	31.8	Cholesterol and cholestanol
7	41.0–46.0	326	
8	46.0–48.2	368	Cholestanol
9	48.2–49.7	357	
10	49.7–51.1	370	
11	51.1–52.5	356	
12	52.5–58.0	366	

Since the original chromatographed mixture consisted of nearly equal amounts of carrier cholestanol and liver sterol (over 90 per cent cholesterol), the C^{14} specific activities of the two separated sterols were proportional to their respective total activities. Hence it can be calculated that *only 0.2 per cent* of the activity of the cholesterol-cholestanol fraction remains associated with cholesterol. In view of the difficulties involved in the complete separation of these two sterols (10), the remaining activity can also be attributed to cholestanol.

When the total activities of the crude digitonide and the purified cholestanol fractions are compared, it is noted that only 66 per cent of the activity of the crude material is accounted for in the cholestanol fraction. Our data indicate the improbability that radiocholestenone contamination or experimental loss is responsible for all of this missing activity. Anker and

XUM

Bloch (1) have shown that the labeled sterol mixture, after deuteriocholestenone feeding, does not contain any appreciable quantity of coprostanol. There thus remains the possibility that under our experimental conditions cholestenone is converted to other digitonin-precipitable substances.

SUMMARY

1 hour after injection of carbon-labeled cholestenone into the portal vein of the rat, the digitonin-precipitable fraction of the liver non-saponifiable fraction contained 53 per cent of the injected activity. Chromatography with carrier cholestanol indicated that 66 per cent of this activity was associated with the cholesterol and cholestanol fractions and specifically with cholestanol. 1.2 per cent of this activity remained with the cholesterol fraction, but further purification of the cholesterol reduced its activity to an insignificant level. It is concluded that cholestenone is not converted to cholesterol under these conditions.

BIBLIOGRAPHY

1. Anker, H. S., and Bloch, K., *J. Biol. Chem.*, **178,** 971 (1949).
2. Turner, R. B., *J. Am. Chem. Soc.*, **72,** 579 (1950).
3. Cox, J. D., Turner, H. S., and Warne, R. J., *J. Chem. Soc.*, 3167 (1950).
4. Heard, R. D. H., and Ziegler, P., *J. Am. Chem. Soc.*, **73,** 4036 (1951).
5. Gora, E. K., and Hickey, F. C., *Anal. Chem.*, **26,** 1158 (1954).
6. Meier, J. R., Siperstein, M. D., and Chaikoff, I. L., *J. Biol. Chem.*, **198,** 105 (1952).
7. Haslewood, G. A. D., *Biochem. J.*, **41,** 639 (1947).
8. Bergmann, W., *J. Biol. Chem.*, **132,** 471 (1940).
9. Fieser, L. F., *J. Am. Chem. Soc.*, **75,** 5421 (1953).
10. Frantz, I. D., Jr., and Bucher, N. L. R., *J. Biol. Chem.*, **206,** 471 (1954).
11. Stokes, W. M., Hickey, F. C., and Fish, W. A., *J. Am. Chem. Soc.*, **76,** 5174 (1954).
12. Idler, D. R., and Baumann, C. A., *J. Biol. Chem.*, **195,** 623 (1952).
13. Schoenheimer, R., *Z. physiol. Chem.*, **192,** 86 (1930).

SERINE METABOLISM IN A MUTANT STRAIN OF ESCHERICHIA COLI STRAIN K-12*

By JOSEPHINE O. MEINHART† and SOFIA SIMMONDS

(*From the Department of Biochemistry, Yale University, New Haven, Connecticut*)

(Received for publication, August 30, 1954)

The metabolic interconversion of serine and glycine was demonstrated first in the intact rat (1–3). That a similar metabolic relationship between serine and glycine also exists in *Escherichia coli* was implicit in the observation that certain amino acid auxotrophs (*i.e.*, mutant strains requiring for growth an exogenous source of a specific amino acid) can grow in media containing either serine or glycine (4–6), and evidence that this relationship exists in a wild type *E. coli* has been obtained with the aid of isotopically labeled compounds (7). Recently, Abelson (8) has reported that, on the basis of experiments with the "isotopic competition" technique, wild type *E. coli* strain B can form glycine from serine but does not carry out the reverse reaction.

A study of the metabolism of serine and glycine in *E. coli* strain K-12 has been made possible by the availability of an auxotroph that grows only in the presence of exogenous serine (strain S) and an auxotroph that can grow in the presence of either serine or glycine (strain S/G). Strain S also provides a test organism for an investigation of serine metabolism under conditions in which both the synthesis of serine from glucose carbon and ammonia nitrogen and the formation of serine from glycine are prevented by a "genetic block." The present communication reports on several aspects of the amino acid metabolism of these two mutants of *E. coli* strain K-12 and of two serine-glycine auxotrophs of *E. coli* strain W.[1] The latter pair of mutants are strain 42-24, which has an absolute requirement for serine or glycine, and strain 55-5, which requires serine or glycine when it is cultured at 35° but will grow in the basal medium at 25°.

* This study was aided by a grant from the Rockefeller Foundation. The data presented in this paper were taken, in part, from a dissertation presented by Josephine O. Meinhart to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.

† National Science Foundation Fellow. Present address, Department of Biochemistry, University of Washington School of Medicine, Seattle.

[1] Strain S (W-1977) and strain S/G (W-1975) were kindly supplied by Dr. Joshua Lederberg, Department of Genetics, University of Wisconsin, and strains 42-24 and 55-5 by Dr. Bernard D. Davis, Department of Pharmacology, New York University College of Medicine.

Growth Response to Serine and Glycine

The growth tests were carried out by the methods described previously (9). Test compounds were added to the basal medium (pH 6.7) prior to sterilization in the autoclave, and glucose was added to the autoclaved medium to provide a final concentration of 0.5 per cent sugar; all tests were run in Evelyn colorimeter tubes containing a final volume of 7 ml.

The *E. coli* mutants were carried routinely on "complete agar" slants (9), and inocula for growth tests consisted of aqueous suspensions of cells harvested from the surface of 16 to 24 hour slants. Two types of inocula were used: A "small" inoculum contained approximately 2×10^6 cells and a "large" inoculum approximately 4×10^7 cells. Unless otherwise stated, the cultures were incubated on a shaker at 35°, and growth was followed as a function of time for each concentration of test compound by absorbancy (optical density) measurements in an Evelyn colorimeter (filter No. 540).

For both strain S and S/G, the "lag time" of the growth cycle was 12 to 15 hours when a small inoculum was used and 4 to 6 hours when a large inoculum was used. In a medium supplemented with the requisite amino acids, maximal (*i.e.*, final) growth on all concentrations of the test compounds was attained in about 24 hours from a small inoculum and in about 15 hours from a large inoculum. In this medium, the "generation time" was about 1.5 hours for strain S and about 2 hours for strain S/G.

In order to compare the growth-promoting action of different test compounds, the concentration of each compound required to give a maximal optical density of 0.15 was estimated from a "concentration curve" obtained by plotting the optical densities at the end of the growth cycles (*i.e.*, the maximal optical density) against the concentrations of the test compound used. An optical density of 0.15 corresponds to approximately one-third of the *optimal* growth of which the auxotrophs are capable in a medium supplemented with non-limiting concentrations of L-serine. In the discussion to follow, the concentration of test compounds will be given as millimoles per liter (mM).

The growth response of the strains to L-serine and glycine is illustrated by the data in Table I. Under the conditions generally employed in this laboratory to determine the growth factor requirements of an amino acid auxotroph, *i.e.* with a small inoculum, strain S does not use glycine in place of serine; incubation of a small inoculum for 60 hours in a medium that is 1 mM with respect to glycine does not induce any detectable growth. When a large inoculum is tested, glycine (0.6 mM) permits a doubling of the optical density of the culture in 25 hours (Fig. 1, *A*). However, in many of the experiments with large inocula incubated in the presence of glycine, "reversion" of strain S (as determined by ability to grow in the unsupple-

mented basal medium) occurred about 10 hours after inoculation of the medium. This tendency toward reversion was only very rarely encountered when strain S was incubated in a medium devoid of both glycine and serine.

TABLE I

Growth Response to L-Serine and Glycine

Unless noted otherwise, the cultures were inoculated with cells harvested from 16 to 24 hour complete agar slants and were incubated on a shaker at 35°.

Strain	mM concentration of test compound required for maximal optical density of 0.15						Remarks
	L-Serine		Glycine		L-Serine + glycine*		
	Small inoculum	Large inoculum	Small inoculum	Large inoculum	Small inoculum	Large inoculum	
S	0.43	0.75	No growth†	No growth†	0.32 of each	0.57 of each	
S/G	0.46	0.75‡	0.37	0.37	0.21 of each	0.34 of each	
	1.84						Stationary incubation
	0.48	No growth†	0.35	0.44			Inoculum from 6.5 hr. complete agar slant
	0.50	No growth†	0.36	0.44			Inoculum from 12 hr. complete agar slant
	0.30	0.40§					Inoculum from 24 hr. culture in basal medium + glycine
	0.46	0.39§					Inoculum from 24 hr. culture in basal medium + L-serine
42-24	0.56	0.40§	0.30	0.35			
	0.95						Stationary incubation
55-5		0.25§		0.11			

* Equimolar mixture of amino acids.

† The highest concentration tested was 1.0 mM (see the text).

‡ The lag time of the culture was about 6 hours.

§ The lag time of the culture was 2 to 3 hours.

The growth response of strain S in media containing mixtures of L-serine and glycine (Table I and Fig. 1) suggests that the presence of glycine reduces the requirement for serine. As may be seen from the curves in Fig. 1, glycine does not exert a typical "sparing effect" on serine; rather, it appears to permit a slow but continuous increase in the optical density of cultures that have exhausted their exogenous supply of serine. Viable cell counts were not made during the second phase of the growth cycle on

the serine-glycine mixtures, and it may only be inferred that the observed increase in optical density was a result of cell multiplication. In the experiments illustrated in Fig. 1, the cells present in the cultures at the end of the incubation did not grow when transferred to the unsupplemented basal medium; in many similar experiments, however, reversion occurred in the serine-glycine media. The results of bioassays indicate that practically all the glycine initially added to the basal medium is still present at the time of maximal growth on serine-glycine mixtures;[2] it is difficult, therefore, to offer any reasonable explanation of the glycine effect. Taken

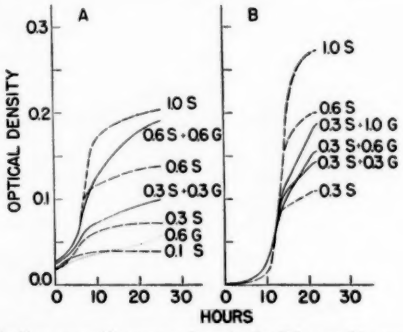


FIG. 1. Representative growth curves for strain S in media containing L-serine (*S*, dash line), glycine (*G*, dotted line), or mixtures of L-serine and glycine (solid lines). The initial concentrations of the test compounds, in millimoles per liter, are indicated for each curve. *A*, experiment with large inocula. Optical density measurements made at approximately 2 hour intervals for the period 0 to 15 hours; a final measurement made at 25 hours. *B*, experiment with small inocula. Optical density measurements made at approximately 2 hour intervals during the period from 10 to 22 hours.

together, all the data permit the conclusion that strain S does not initiate rapid growth when only glycine is provided in the medium, and that the apparent response to glycine depends on the number and metabolic state of the cells exposed to this amino acid.

It is of interest that, in spite of the obvious difference in the biosynthetic abilities of strains S and S/G, the two auxotrophs have the same requirement for L-serine. When it is grown from a small inoculum, strain 42-24 shows a serine requirement like that of the mutants of strain K-12. The glycine requirements of strains S/G and 42-24 also are similar. That strain 55-5 has a much lower requirement for serine and for glycine may be a consequence of the ability of this auxotroph to grow in the absence of an exogenous source of either amino acid at 25°.

[2] Meinhart, J. O., and Simmonds, S., unpublished data.

At the start of this study of serine metabolism, it was appreciated that the requirement for serine might be fairly high if a portion of the exogenous serine is deaminated by the bacteria (10–12) and thus becomes unavailable for use in the anabolic processes associated with cell multiplication. The serine requirement has been found to be markedly influenced by a number of factors, all of which are believed to exert an effect on the activity of the bacterial "serine deaminase." As reported by Gale and Stephenson (10), the ability of resting cells of *E. coli* to deaminate serine is increased by growing broth cultures anaerobically rather than aerobically, and by harvesting the cells from 11 hour cultures rather than from 24 hour cultures. The data in Table I are in complete accord with these findings. The observation that the serine requirement of the large inoculum of strain 42-24 was relatively low is probably the result of the short lag time of the culture during the growth test. A similar explanation may be applied to the relatively low serine requirement of the large inoculum of strain S/G harvested from the liquid medium supplemented with serine; thus the expected increase in the serine deaminase activity of the cells used for the inoculum is masked by the fact that growth was initiated so soon after inoculation. On the other hand, the deaminase activity of large inocula harvested from 6.5 and 11 hour slants was probably so great that all the serine in the medium was destroyed before growth could start.

Under all experimental conditions the serine requirements of strains S and S/G are of an entirely different order of magnitude from the amino acid requirements of other auxotrophs of strain K-12 that have been studied in this laboratory; mutants which require L-cystine (13), L- or DL-methionine (13), L-proline (14), L-proline or L-glutamic acid (14), L-leucine (15), L-phenylalanine (16), or L-tyrosine (16) will grow, on stationary incubation, to an optical density of about 0.15 in the presence of 0.01 to 0.04 mM concentrations of the required amino acids.

Resting cell suspensions of strains S and S/G have been found to be capable of deaminating both L-serine and glycine (Table II). Presumably these mutants, like other strains of *E. coli* (11, 12), deaminate L-serine by an anaerobic reaction in which pyruvic acid is the final product; the deamination of glycine probably is an aerobic reaction and yields glyoxylic acid (18). The deaminase activity of strain S toward serine is much less than that of strain S/G, and the activity of the latter strain is clearly an "adaptive" property of the organism. The results of preliminary experiments in which strains S and S/G were harvested from cultures grown under partially anaerobic conditions suggest that the serine deaminase activity of the resting cells is greatly increased by this treatment.[2]

The data in Table II also may be taken to indicate that "glycine deaminase" is an adaptive enzyme. However, even with resting cells of

strain S/G grown in media containing glycine, significant deamination of glycine does not become apparent until 15 or 20 hours after the start of the incubation period. A similar slow deamination of glycine during the first

TABLE II

Deamination of L-*Serine and Glycine by Resting Cells*

The resting cell suspensions were harvested from cultures of strain S or S/G grown in 500 ml. flasks containing 100 ml. of the basal medium and 100 μmoles of L-serine or glycine. The flasks were incubated on a shaker at 35° for 24 hours, and the cells were collected by centrifugation, were washed with 0.9 per cent saline, and were suspended in water. The deamination experiments were carried out at 35° in a total volume of 4 ml. containing 15 μmoles of L-serine or glycine and 1 ml. of cell suspension in an inorganic salt, phosphate buffer (pH 6.7) solution composed of Na_2SO_4, K_2HPO_4, KH_2PO_4, $MgSO_4$, and trace elements in the concentrations used for the growth medium.

Time	Strain S, grown on serine			Strain S/G, grown on serine			Strain S/G, grown on glycine		
	Bacterial nitrogen*	Deamination†		Bacterial nitrogen*	Deamination†		Bacterial nitrogen*	Deamination†	
		Serine	Glycine		Serine	Glycine		Serine	Glycine
hrs.	γ	*per cent*	*per cent*	γ	*per cent*	*per cent*	γ	*per cent*	*per cent*
0	73	1	0	72	0	1	56	3	2
3		2	1		13	2		6	2
6		9	3		28	6		1	0
9		13	11		40	5		5	3
12		16	8		57	7		18	5
10	32	5	5	45	21	3	36	17	5
12		3	4		38	5		19	6
15		6	8		38	6		22	6
18		7	9		49	11		29	9
21		6	10		57	10		37	14
24		10	15		71	20		42	22
34		17	63		85	61		46	100
37		16	93		86	85		50	100

* The total bacterial nitrogen in each cell suspension was determined by the micro-Kjeldahl method.

† The per cent deamination was calculated from the amount of ammonia formed; ammonia was determined by a modified Conway procedure (17). All values were corrected for "cell blanks."

20 hours of incubation has been found for resting cells of a proline auxotroph of *E. coli* strain K-12, and seems to be characteristic of the parent wild type (19).

The data in Table I show that glycine and serine are equally effective in promoting the growth of strain S/G, provided that the experimental con-

ditions are such as to minimize the deamination of serine. The relative slowness with which resting cells deaminate glycine explains why the requirement for glycine in growth tests is the same for large and small inocula, and it may be inferred that a concentration of about 0.35 mM represents that amount of glycine (or serine) required for the processes of cell growth and multiplication in a culture whose maximal optical density is 0.15. The fact that this amount of glycine is so much greater than the amounts in which other amino acids are required may be a reflection of the importance of glycine as a precursor in the biosynthesis of cell constituents such as purines, pyrimidines, and porphyrins.

Up to the present time, no substances (other than serine or glycine peptides) have been found to replace the serine and glycine which must be supplied in the medium for the growth of strains S and S/G. When added to the basal medium, Bacto yeast extract has a slight growth-promoting action for strain S and is an excellent source of the growth factors required by strain S/G. However, yeast extract also has an inhibitory effect on the growth response of these organisms to L-serine. Yeast extract does not inhibit the growth of strain S/G in the presence of glycine, but it does not permit strain S to grow in a medium containing glycine in place of serine. Experiments to test the ability of amino acids (other than glycine) to spare the serine requirements of the two mutants have given uniformly negative results, and no amino acid has been found to spare the glycine requirement of strain S/G. It may be concluded, therefore, that in so far as amino acids are concerned the auxotrophs require only an exogenous source of serine or glycine; it is not clear whether growth factors other than amino acids can partially satisfy these requirements.

Isotope Experiment

In order to obtain additional information concerning the relationship between serine and glycine in the metabolism of strain S, and to determine whether exogenous serine is the sole source of the serine in the bacterial proteins, an isotope experiment was carried out in which strain S was grown in the presence of uniformly labeled glucose-C^{14} and unlabeled L-serine. The medium used in this experiment differed from the medium used in growth tests in two respects: (1) The glucose content was 0.1 per cent rather than 0.5 per cent (the former concentration having been found to be the lowest that would permit maximal growth on 1 mM L-serine, which was to be the growth-limiting factor), and (2) L-asparagine was omitted from the medium to eliminate any reactions in which asparagine-C^{12} might serve as a source of carbon for amino acid synthesis.

A small inoculum (about 2×10^9 cells) was added to 8 liters of medium containing 8 gm. of radioactive glucose (1 μc.) and 840 mg. of L-serine.

The culture was incubated at 35° under forced aeration until maximal growth was attained (36 hours). The cells were collected by centrifugation and were washed well with 0.9 per cent saline, water, absolute ethanol, and absolute ether; the residue, dried *in vacuo* over P_2O_5, weighed 1.8 gm. This bacterial powder was hydrolyzed in 6 N hydrochloric acid, and the hydrolysate was subjected to chromatographic separation on a column of Dowex 50 resin according to the method of Stein and Moore (20). The purity of each amino acid sample isolated was checked by paper chromatography. The amino acids isolated and analyzed for C^{14} are listed in Table III.

The complete absence of radioactivity in the isolated serine shows that strain S is completely unable to synthesize serine from glucose carbon. Since both the exogenous serine and the bacterial serine were non-radioactive, any bacterial glycine derived directly from serine by a cleavage between the α- and β-carbon atoms (1) would also be non-radioactive. From the amount of radioactivity in the isolated glycine, it may be calculated that at least one-third of the carbon in the glycine came from glucose by pathways that exclude the intermediate formation of serine. One such pathway might involve the cleavage of threonine to give glycine and a 2-carbon fragment (21); data suggesting the presence of this pathway in *E. coli* have been reported by Ravel *et al.* (22) and by Abelson (8). A second possible pathway of glycine synthesis from glucose is via the formation of glycolaldehyde, glycolic acid, and glyoxylic acid (23, 24). Although this pathway has not been reported to exist in *E. coli*, the last step in the synthesis, the conversion of glyoxylic acid to glycine by a transamination reaction, could be catalyzed by the glycine-α-ketoglutaric acid transaminase that has been demonstrated to be present in dried cells of this organism (25).

The data in Table III show that all the carbon of glutamic acid and essentially all the carbon of arginine were derived from glucose-C^{14}. The biosynthesis of the ornithine moiety of arginine from glutamic acid has been established for *E. coli* strain B (8, 26–28), and the same biosynthetic pathway is assumed to be followed in strain S. It is of interest that the guanidino carbon of arginine must have arisen mainly from glucose-C^{14} and not from $C^{12}O_2$ in the atmosphere or formed from serine.

The synthesis of glutamic acid from glucose via the tricarboxylic acid cycle (28) obviously did not involve any dilution of C^{14} by carbon arising either from atmospheric CO_2 or from serine. This would suggest that unlabeled pyruvic acid formed by the deamination of the exogenous serine did not "mix" with radioactive pyruvic acid formed from glucose. Support for the view that such mixing did not occur is found in the fact that the bacterial alanine was practically devoid of C^{14}. If serine is deaminated prior to the initiation of bacterial growth, and if the amount of pyruvic acid formed is very large compared with the amount of pyruvic acid arising

from the degradation of glucose during this period, the pyruvic acid immediately available to the cells would be essentially free of C^{14}. In effect,

TABLE III

Radioactivity of Isolated Amino Acids

Amino acid*	Amount plated	Radioactivity		Calculated radioactivity $\left(\frac{a}{b} \times 100\right)$
		Observed† (*a*)	Calculated‡ (*b*)	
	mg.	*c.p.m.*	*c.p.m.*	*per cent*
Serine	1.4	0§	135	0
Glycine	2.0	59	180	33.3
Glutamic acid·HCl	2.0	185	183	100
Arginine·HCl	4.1	390	395	99
Alanine	7.0	9	798	1
Cystine	1.2	2	101	2
Aspartic acid	1.5	133	152	88
	3.0‖	269	305	88
Threonine	2.8¶	53	318	17
	2.1	62	239	25
Valine	0.6	81	87	93
Leucine	1.8	251	279	90

* Glutamic acid hydrochloride, arginine hydrochloride, and glycine were isolated directly from the appropriate fractions of the Dowex 50 eluent. A fraction containing serine, threonine, and aspartic acid was rechromatographed on Dowex 1 resin (acetate form) to free it of aspartic acid; the resultant threonine-serine mixture was resolved by paper chromatography with water-saturated phenol as the developing solvent. A second sample of aspartic acid was isolated directly from the Dowex 50 eluent. Alanine was freed of traces of glycine and ammonium salts by paper chromatography with butanol-water-pyridine (4:1:1) as the solvent. Leucine and cystine were separated from each other by paper chromatography with butanol-water-acetic acid (4:1:1) as the solvent; this solvent was also used to free valine of traces of proline by paper chromatography.

† Radioactivity, corrected for background, as determined in a gas flow counter in the Geiger region. Except for serine, alanine, and cystine, the samples were counted to a probable error of 2 per cent or less; alanine and cystine were counted to a probable error of about 5 per cent.

‡ Calculated on the basis that all the carbon atoms are derived from glucose-C^{14} which had a specific activity of 3.38 c.p.m. per 12 γ of glucose carbon.

§ The samples of serine contained no radioactivity in any of several determinations made.

‖ Sample isolated directly from the Dowex 50 eluent that contained the major portion of the bacterial aspartic acid.

¶ This sample may have been contaminated with serine.

the pyruvic acid-C^{12} formed from serine during the lag period of the growth cycle may be considered an "isotopic competitor" of glucose-C^{14}. Such a

competition between pyruvic acid and glucose in *E. coli* strain B has been reported to suppress the utilization of glucose carbon for alanine synthesis (8).[3] In strain B, exogenous serine suppresses to some extent the utilization of glucose carbon for the biosynthesis of alanine and suppresses almost completely the utilization of glucose carbon for the biosynthesis of cystine (8). The latter phenomenon obtains in strain S, in which the cystine, like the alanine, had only a trace of radioactivity. The mechanism whereby serine carbon is utilized for cystine synthesis in *E. coli* is not clear; none of the available data (13) concerning cystine and cysteine metabolism in strain K-12 supports the view that this strain of *E. coli* is capable of forming cysteine by the condensation of serine and homocysteine to yield cystathionine followed by the cleavage of the thio ether, as occurs in mammals (29). However, the presence of cysteine desulfhydrase in *E. coli* is known (30), and the reversal of the catabolic action of this enzyme system may explain how cysteine can be formed from ammonia, inorganic sulfide, and pyruvic acid (31). If this pathway is followed, the pyruvic acid formed from serine is utilized for cysteine synthesis without significant dilution by pyruvic acid formed from glucose.

Although the isotope data cited above suggest that the carbon compounds formed by the degradation of serine and glucose did not enter a common metabolic pool, the radioactivity of the aspartic acid indicates that such a conclusion is not entirely warranted. The dilution of C^{14} in the biosynthesis of aspartic acid is not very large, but it is sufficient to indicate that some of the carbon supplied as serine can be used in the formation of oxalacetic acid. However, it would appear that the oxalacetic acid so formed is not in equilibrium with the component acids of the tricarboxylic acid cycle, since there is no dilution of C^{14} in the glutamic acid. It is possible, also, that, in the early stages of the growth cycle, aspartic acid was synthesized directly from the pyruvic acid arising from serine, and only later was pyruvic acid arising from glucose used for this synthesis.

If it is assumed that, in so far as the biosynthesis of aspartic acid, alanine, and cystine is concerned, serine functions only as a precursor of pyruvic acid, then the data for the relative radioactivity of aspartic and glutamic acids isolated from strain S are at variance with the data of Abelson (8); in strain B, pyruvic acid suppressed the utilization of glucose carbon for the synthesis

[3] In comparing the data for strain S with those obtained by the method of isotopic competition for strain B, it should be noted that, in the experiment with the serine auxotroph of *E. coli* strain K-12, an inoculum composed of cells in the "resting state" went through a complete growth cycle consisting of about eight generations in a medium that contained, per ml., 1 mg. of glucose and 0.105 mg. of L-serine; in the experiments with strain B, a wild type strain of *E. coli* was studied under conditions in which cells in logarithmic growth underwent one cell division in a medium that contained, per ml., 0.2 mg. of glucose and 0.1 or 0.2 mg. of competitor (8).

of both aspartic and glutamic acids. Abelson (8) also reported that pyruvic acid suppressed aspartic acid and threonine synthesis to the same extent. The threonine isolated from strain S had much less C^{14} than the aspartic acid; therefore, either strain S did not form all its threonine from aspartic acid or, if it did so, the aspartic acid used for threonine synthesis was not in equilibrium with the aspartic acid incorporated *per se* into the bacterial proteins. Cohen and Hirsch (32, 33) have shown that several strains of *E. coli*, including K-12, are capable of converting aspartic acid to threonine, but the extremely low radioactivity of the threonine suggests that another pathway of threonine synthesis exists in strain S. One likely mechanism is the condensation of glycine with a 2-carbon unit; *i.e.*, a reversal of the process by which threonine is cleaved to form glycine (21). The formation of threonine by yeast incubated in the presence of glycine and glucose was observed several years ago (34), and this pathway would readily explain the data obtained with strain S, since the bacterial glycine was found to be low in C^{14} and a 2-carbon unit derived in large part from serine via pyruvic acid would likewise contain little radioactivity.

The valine and leucine isolated from strain S (Table III) were highly radioactive; thus, these amino acids appear to have been synthesized primarily from glucose-C^{14}. The data for leucine and valine are in agreement with those of Abelson (8).

From the results obtained in the isotope experiment with strain S, it is clear that essentially all the bacterial alanine and cystine and large fractions of the glycine and threonine were formed from carbon supplied as serine rather than from glucose carbon. It might be expected that the addition of these amino acids to the basal medium would spare the growth requirement of the strain for serine. The data from the growth tests indicate that no amino acid (with the possible exception of glycine) has such a sparing action. This is not surprising if the utilization of serine carbon in the biosynthesis of other amino acids is due primarily to the fact that serine is rapidly converted to pyruvic acid, which is the obligatory intermediate in the biosynthetic reactions leading from glucose to the amino acids mentioned.[4] The true rôle of serine as an obligatory intermediate in amino acid synthesis can be determined only under conditions in which serine does not also serve as a source of pyruvic acid.[5]

[4] In this connection, it may be noted that pyruvic acid (1.0 mM) neither replaces nor spares the serine required for growth by strain S. On the other hand, L-serine has been found to serve as the sole carbon source for the growth of strain K-12 and of strain S/G cultured in a medium containing glycine.

[5] After this manuscript had been submitted for publication, the ability of certain amino acids, including L-alanine, L-glutamic acid, L-methionine, and L-threonine, to exert a sparing effect on the serine requirement of strain S was reinvestigated. In confirmation of earlier findings, alanine, glutamic acid, and methionine were found

SUMMARY

Several aspects of the metabolism of L-serine and glycine by a serine auxotroph (strain S) and by several serine-glycine auxotrophs (strains S/G, 42-24, and 55-5) of *Escherichia coli* have been investigated. The apparent growth requirements of the auxotrophs for serine and glycine are extremely high. A study of the ability of strains S and S/G to deaminate L-serine and glycine indicates that the high requirement for serine may be explained only in part by the deamination of serine prior to the utilization of the amino acid for growth.

Data from an experiment in which strain S was grown in the presence of uniformly labeled glucose-C^{14} and unlabeled serine show that serine can serve as a carbon source for the biosynthesis of glycine, but another pathway of glycine synthesis that does not involve serine as an obligatory intermediate is also operative. Other aspects of the amino acid interrelationships in this auxotroph are discussed.

BIBLIOGRAPHY

1. Shemin, D., *J. Biol. Chem.*, **162,** 297 (1946).
2. Winnick, T., Moring-Claesson, I., and Greenberg, D. M., *J. Biol. Chem.*, **175,** 127 (1948).
3. Sakami, W., *J. Biol. Chem.*, **176,** 995 (1948).
4. Roepke, R. R., Libby, R. L., and Small, M. H., *J. Bact.*, **48,** 401 (1944).
5. Davis, B. D., and Maas, W. K., *J. Am. Chem. Soc.*, **71,** 1865 (1949).
6. Wright, B., *Nature*, **168,** 1087 (1951).
7. Cutinelli, C., Ehrensvärd, G., Reio, L., Saluste, E., and Stjernholm, R., *Acta chem. Scand.*, **5,** 353 (1951).
8. Abelson, P. H., *J. Biol. Chem.*, **206,** 335 (1954).
9. Simmonds, S., Dowling, M. T., and Stone, D., *J. Biol. Chem.*, **208,** 701 (1954).
10. Gale, E. F., and Stephenson, M., *Biochem. J.*, **32,** 392 (1938).
11. Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, **151,** 273 (1943).
12. Maas, W. K., and Davis, B. D., *J. Bact.*, **60,** 733 (1950).
13. Simmonds, S., *J. Biol. Chem.*, **174,** 717 (1948).
14. Simmonds, S., and Fruton, J. S., *J. Biol. Chem.*, **174,** 705 (1948).
15. Simmonds, S., Tatum, E. L., and Fruton, J. S., *J. Biol. Chem.*, **170,** 483 (1947).
16. Simmonds, S., Tatum, E. L., and Fruton, J. S., *J. Biol. Chem.*, **169,** 91 (1947).
17. Johnston, R. B., Mycek, M. J., and Fruton, J. S., *J. Biol. Chem.*, **185,** 629 (1950).
18. Janke, A., and Tayenthal, W., *Biochem. Z.*, **289,** 76 (1936–37).
19. Stone, D., Thesis, Yale University (1951).

to be totally inactive in this respect. Threonine, however, proved capable of sparing serine in growth tests with small inocula, although it did not serve as a growth factor in a medium devoid of serine. Further tests with mixtures of serine and threonine showed that the initial growth of the culture occurs at the expense of the exogenous serine and that the threonine disappears from the medium after the exogenous serine has been exhausted. It may be inferred, therefore, that the presence of exogenous threonine would not repress the utilization of serine carbon for the biosynthesis of bacterial threonine; rather, the bacteria appear to be capable of utilizing threonine to form serine under the conditions of these tests.

20. Stein, W. H., and Moore, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14,** 179 (1949).
21. Meltzer, H. L., and Sprinson, D. B., *J. Biol. Chem.*, **197,** 461 (1952).
22. Ravel, J. M., Eakin, R. E., and Shive, W., *J. Biol. Chem.*, **172,** 67 (1948).
23. Weinhouse, S., and Friedmann, B., *J. Biol. Chem.*, **191,** 707 (1951).
24. Weissbach, A., and Sprinson, D. B., *J. Biol. Chem.*, **203,** 1023, 1031 (1953).
25. Feldman, L. I., and Gunsalus, I. C., *J. Biol. Chem.*, **187,** 821 (1950).
26. Vogel, H., *Proc. Nat. Acad. Sc.*, **39,** 578 (1953).
27. Maas, W. K., Novelli, G. D., and Lipmann, F., *Proc. Nat. Acad. Sc.*, **39,** 1004 (1953).
28. Abelson, P. H., Bolton, E., Britten, R., Cowie, D. B., and Roberts, R. B., *Proc. Nat. Acad. Sc.*, **39,** 1020 (1953).
29. Binkley, F., and du Vigneaud, V., *J. Biol. Chem.*, **144,** 507 (1942).
30. Desnuelle, P., and Fromageot, C., *Enzymologia*, **6,** 80 (1939).
31. Smythe, C. V., and Halliday, D., *J. Biol. Chem.*, **144,** 237 (1942).
32. Cohen, C. N., and Hirsch, M. L., *Compt. rend. Acad.*, **236,** 1302 (1953).
33. Hirsch, M. L., and Cohen, C. N., *Compt. rend. Acad.*, **236,** 2338 (1953).
34. Rossi, A., and Cennamo, C., *Boll. Soc. ital. biol. sper.*, **20,** 194 (1945).

16-SUBSTITUTED STEROIDS

XI. ESTRADIOL-3,16β

By MAX N. HUFFMAN and MARY HARRIET LOTT

(From the Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma)

(Received for publication, September 29, 1954)

Recently we described (1) the preparation of 1,3,5(10)-estratrien-3-ol-16-one, which differs from natural estrone only in possessing the ketonic oxygen at position C_{16} instead of position C_{17}. Inasmuch as estradiol-3,17β is generally accepted as the estrogenic hormone of the ovary, we thought it of importance to prepare its spatial analogue in the 16-oxygenated series, estradiol-3,16β, in order to compare the biological properties of the latter with those of the hormone.

Estrone-16 may be readily reduced with hydrogen and Adams' catalyst to furnish estradiol-3,16β. This diol is even more easily prepared by the sodium borohydride reduction of estrone-16. However, in the partial synthesis of estrone-16 by the mild Clemmensen reduction of 16-ketoestradiol, as previously described (1), often estrone-16 is not obtained, an intractable mixture of estrone-17 and estrone-16 resulting instead. Nevertheless, it is possible to isolate estradiol-3,16β from this intractable mixture if the latter is reduced to the diol stage with sodium borohydride, for it has been found that estradiol-3,16β does not furnish a digitonide with digitonin, as does natural estradiol (2). It is also possible to separate the estradiol-3,16β from estradiol-3,17β by fractional crystallization.

Estradiol-3,16β was characterized as the 3-monobenzoate, the 3,16-diacetate, the 3-methyl ether, and the 3-methyl ether-16-acetate.

In the Allen-Doisy assay for estrogenic activity in the adult sprayed rat, estradiol-3,16β showed the same biological potency at a level of 83 γ as did estrone U. S. P. at a level of 0.83 γ (75 per cent of twenty animals with each compound). Estradiol-3,16β inhibited gonadotropic hypersecretion in the parabiotic rat at a total dose of 40 γ.[1]

Our assignment of the β configuration to the carbinol epimer, which is formed preferentially in the reduction of a 16-ketosteroid, will be discussed at length in a forthcoming publication.

[1] These bioassays were performed by the Endocrine Laboratories of Madison, Wisconsin, under the direction of Dr. Elva Shipley.

XUM

EXPERIMENTAL[2]

Preparation of Estradiol-3,16β

By Hydrogenation of Estrone-16—To a solution of 250 mg. of pure 1,3,5-(10)-estratrien-3-ol-16-one (estrone-16) in 100 ml. of 0.5 N sodium hydroxide were added 400 mg. of Adams' catalyst, and the whole was shaken under 15 pounds pressure of hydrogen for 12 hours at room temperature. The reaction mixture was then filtered free of catalyst, and the filtrate acidified with concentrated hydrochloric acid, diluted with water, and extracted with ethyl ether. The ether, after having been washed twice with water, was evaporated to yield a crystalline residue. To remove any remaining ketones, the dried residue was allowed to react with carboxymethoxylamine hemihydrochloride in the usual fashion. The non-ketonic fraction was recrystallized from aqueous methanol, from acetone-cyclohexane, and from aqueous acetone to yield 123 mg. of flat needles of estradiol-3,16β melting at 224–226°, with slight decomposition.

The 123 mg. of estradiol-3,16β, as above, were dissolved in 60 ml. of 0.5 N sodium hydroxide with the aid of heat, and, after cooling, 1.0 ml. of benzoyl chloride was added. The stoppered mixture was shaken vigorously for 10 minutes and allowed to remain at room temperature overnight. The precipitated benzoate was filtered, washed with dilute sodium hydroxide, washed with water, and then dried *in vacuo*. After having been recrystallized once from aqueous ethanol, once from aqueous methanol, twice from Skellysolve B, and twice from aqueous methanol (treatment with charcoal), the yield of felt-like needles of *3-benzoxyestra-1,3,5(10)-trien-16β-ol* was 83 mg., m.p. 145–146°.

$C_{25}H_{28}O_2$.	Calculated.	C 79.75, H 7.50
	Found. (*a*)	" 79.81, " 7.50
	(*b*)	" 79.88, " 7.57

Pure 3-benzoxyestra-1,3,5(10)-trien-16β-ol, 69 mg., was dissolved in 20 ml. of methanol plus 10 ml. of 2 N sodium hydroxide. The solution was refluxed for 1 hour; then 2.0 ml. of acetic acid and 5 ml. of water were added and the saponification solution distilled until ebullition had ceased. Upon the slight addition of water, crystallization occurred. The steroid was recrystallized from acetone-cyclohexane, from aqueous acetone, and from aqueous methanol (charcoal). The yield of 1,3,5(10)-estratriene-3,16β-diol was 43 mg., melting at 223–224°, with slight decomposition. Very pure diol melts at 227–227.5°, with slight decomposition.

[2] All melting points listed in this publication are uncorrected. Microanalyses and optical rotations are by Dr. E. W. D. Huffman, Denver.

$C_{18}H_{24}O_2$. Calculated. C 79.37, H 8.88
Found. (*a*) " 79.42, " 8.95
(*b*) " 79.31, " 8.85
$[\alpha]_D^{23}$ +74° (*c* = 1.02 in 95% ethanol)

By Sodium Borohydride Reduction of Estrone-16—Pure estrone-16 (1.25 gm.) was dissolved under a reflux in 100 ml. of methanol, and, after the solution had cooled to 40°, a solution of 1.2 gm. of sodium borohydride in 30 ml. of methanol was stirred in. Stirring was continued at room temperature for a period of 45 minutes, and the steroid then precipitated with 1300 ml. of ice water containing 15 ml. of acetone. After a day at 5°, the diol was collected, washed thoroughly with water, and recrystallized from aqueous methanol. Yield, 1.00 gm., melting at 226–227°.

By Sodium Borohydride Reduction of Mixture, Resulting from Clemmensen Reaction on 16-Ketoestradiol—16-Ketoestradiol (1.65 gm., m.p. 231–233°) was subjected to mild Clemmensen reduction for 5 hours (1) and the reaction worked up as previously described. To the steroidal residue, placed in solution in 80 ml. of methanol, 0.80 gm. of sodium borohydride was added and the mixture swirled for 30 minutes. Then 20 ml. of methanol were used to rinse any unchanged material from the side of the flask and the solution was agitated for another 30 minutes. The excess reducing agent was then expended with acetone, and the solution transferred to a separatory funnel, diluted with 3 per cent sodium bicarbonate, and extracted with ethyl ether. The ether was washed with 3 per cent sodium bicarbonate and with water. After evaporation of the ether, the residue was thoroughly dried, dissolved in 250 ml. of 80 per cent ethanol, and then 5.0 gm. of solid digitonin were mixed in. The mixture was allowed to stand at room temperature for 9 days with mixing each day. There occurred a voluminous white precipitate which, with the solution, was transferred to a separatory funnel and extracted with ethyl ether. The ether was washed exhaustively with water, dried over anhydrous sodium sulfate, and then evaporated to dryness. The residue was crystallized from aqueous methanol and from acetone-Skellysolve B (treatment with charcoal), yielding 459 mg. of steroid which melted at 221–222°. A further recrystallization from aqueous methanol raised the melting point to 226.5–227°, with slight decomposition (429 mg.).

Fractional crystallization was also utilized as a mode of separation. 16-Ketoestradiol (0.8 gm.) was carried through the Clemmensen reduction as described, the remaining residue was crystallized twice from acetone-Skellysolve B (charcoal), once from aqueous methanol, twice from aqueous ethanol, once from acetone-Skellysolve B (charcoal), and once from aqueous acetone, yielding 154 mg. of estradiol-3,16β, which melted at 223.5–225°.

A mixed melting point with authentic 1,3,5(10)-estratrien-3,16β-diol showed no depression of this melting point.

Characterization of Estradiol-3,16β by Derivatives

3-Methoxyestra-1,3,5(10)-trien-16β-ol Acetate—To a solution of 190 mg. of 3-methoxyestra-1,3,5(10)-trien-16-one (m.p. 124–124.5°) (1) in 15 ml. of methanol was added 0.04 gm. of sodium borohydride dissolved in 5 ml. of methanol, and the reaction mixture was stirred mechanically at 20° for 1 hour. Then 1 ml. of acetone was added and the stirring continued for 30 minutes, followed by the addition of 5 ml. of 0.5 N sodium hydroxide and stirring for 15 minutes. The solution was heated gently and water was added to turbidity. After a day at 5° the reduced steroid was collected, dried, and then acetylated in 5 ml. of dry pyridine with 5 ml. of acetic anhydride. The resulting acetate was recrystallized from aqueous methanol to furnish 191 mg. of 3-methoxyestra-1,3,5(10)-trien-16β-ol acetate, m.p. 129.5–130.5°. Another recrystallization from aqueous methanol raised the melting point to 132–133° (175 mg.). Two more recrystallizations from aqueous methanol and treatment with charcoal served only to sharpen the melting point to 132–132.5°.

$C_{21}H_{28}O_3$.	Calculated.	C 76.79, H 8.59
	Found. (*a*)	" 76.83, " 8.58
	(*b*)	" 76.69, " 8.62

The mother liquors from the above crystallizations were extracted with ethyl ether to recover the crude steroid acetate. To this crude acetate were added 89 mg. of pure 3-methoxyestratrien-16β-ol acetate, and saponification was effected by dissolving the combined steroids in 60 ml. of methanol, treating with 30 ml. of 2 N sodium hydroxide, and refluxing for 1 hour. The removal of the methanol by distillation caused the estradiol-3-methyl ether to precipitate. After collection on the filter, there remained 152 mg. of silky needles (m.p. 103.5–104°) of *3-methoxyestra-1,3,5(10)-trien-16β-ol*. Further recrystallizations did not improve the melting point.

3-Methylation of Estradiol-3,16β, Followed by Acetylation—To 38 mg. of estradiol-3,16β (m.p. 225.5–227°) were added 0.80 gm. of anhydrous potassium carbonate and 8 ml. of 90 per cent methanol. The mixture was refluxed until solution was complete, at which time 0.3 ml. of dimethyl sulfate was added. When the reaction had subsided, another 0.3 ml. of dimethyl sulfate was added and refluxing continued until a total time of 45 minutes had elapsed from the time of the first addition. Water (4 ml.) was added and the solution was distilled to turbidity. After several days at 5° the crude methyl ether was collected on the filter and dried *in vacuo*. The steroid was dissolved in 1 ml. of pyridine and treated with 0.5 ml. of acetic

anhydride. The reaction mixture remained a day at room temperature; then the acetate was precipitated with water. After filtering and drying, the steroid was recrystallized from aqueous acetone (charcoal) and twice from aqueous methanol to give 17 mg. of 3-methoxy-16β-acetoxy-1,3,5-(10)-estratriene (m.p. 130–131°). On admixture with authentic 3-methoxy-16β-acetoxy-1,3,5(10)-estratriene, there was no depression of the melting point.

1,3,5(10)-Estratrien-3, 16β-diol Diacetate—49 mg. of pure estradiol-3,16β were acetylated at room temperature with 1.0 ml. of pyridine and 1.0 ml. of acetic anhydride. After 24 hours the diacetate was precipitated with ice water and placed in the ice box for 2 days. The steroid was filtered and recrystallized from 80 per cent methanol, yielding 51 mg. of the diacetate melting at 96–97°. Further recrystallizations did not change the melting point.

$C_{22}H_{28}O_4$. Calculated, C 74.13, H 7.92; found, C 74.31, H 7.88

SUMMARY

The preparation of 1,3,5(10)-estratrien-3,16β-diol (estradiol-3,16β) is described. This compound was characterized as the diacetate, the 3-monobenzoate, the 3-methyl ether-16-acetate, and the 3-methyl ether.

In the Allen-Doisy assay, estradiol-3,16β has 1/100 the estrogenic potency of estrone.

The authors wish to thank Dr. Elva Shipley for performing the bioassays cited in this publication. To the Graduate Research Institute of Baylor University (Dallas) the authors are especially indebted for financial assistance rendered during the course of this investigation.

The technical assistance of Mr. Albert Tillotson in a portion of this research is gratefully acknowledged.

BIBLIOGRAPHY

1. Huffman, M. N., and Lott, M. H., *J. Am. Chem. Soc.*, **75**, 4327 (1953).
2. Wintersteiner, O., *J. Am. Chem. Soc.*, **59**, 765 (1937).

THE FATE OF RADIOACTIVE CHOLESTEROL IN MICE WITH MODIFIED THYROID ACTIVITIES*

By SAMUEL B. WEISS† and WALTER MARX

(From the Department of Biochemistry and Nutrition, School of Medicine, University of Southern California, and the Laboratory Division, Los Angeles County Hospital, Los Angeles, California)

(Received for publication, September 29, 1954)

Recent findings of the authors indicate that the thyroid hormone may stimulate the destruction or conversion of cholesterol in the mouse (1). This effect was demonstrated, however, only under rather specialized conditions, *i.e.*, when a restricted intake of a diet relatively high in cholesterol was employed. The question arose whether the observed phenomenon occurred also under ordinary nutritional conditions. In view of the multiple action of the thyroid hormone on cholesterol metabolism (1, 2), it was felt that this problem could best be approached by studying the fate of radioactive cholesterol in animals with modified thyroid activities. The results of this investigation are reported in the present paper; they indicate that thyroid stimulates the catabolism as well as the fecal excretion of cholesterol.

Methods

Preparation of Radioactive Cholesterol—Labeled cholesterol was prepared by the reduction of the enol acetate of 4-C^{14}-cholesten-3-one[1] with sodium borohydride, and the product obtained was purified as described by Schwenk *et al.* (3). The radioactive sterol had a specific activity of 1.3×10^5 c.p.m. per mg. of C, as measured with a gas flow counter.

Treatment of Animals—Male mice (Swiss colony strain[2]) 6 to 7 weeks old, weighing 20 to 28 gm., were used. Prior to the experimental period, all animals were fed Purina chow supplemented with 0.5 per cent thiouracil[3] for 12 days, and then they were divided into two groups. One group

* This investigation was supported, in part, by a research grant (No. H-1162) from the National Heart Institute, National Institutes of Health, United States Public Health Service.

† Predoctoral Fellow of the Life Insurance Medical Research Fund. Present address, Ben May Laboratory for Cancer Research, University of Chicago, Chicago 37, Illinois.

[1] 4-C^{14}-Cholesten-3-one was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

[2] The mice were obtained from the California Caviary, Los Angeles.

[3] The authors are obliged to the Lederle Laboratories Division, American Cyanamid Company, for providing this material (Deracil).

XUM

continued on the same thiouracil regimen, while the other group was made hyperthyroid by supplementing the thiouracil-containing diet with 0.4 per cent desiccated thyroid,[4] both for a further pretreatment period of 3 days. At this time, 4-C^{14}-cholesterol, dissolved in saline with Tween 20 according to Meier *et al.* (4), was injected into the tail vein of all animals at a level of 1 mg. per 25 gm. of body weight. Then the mice were placed individually in metabolism cages, as previously described (5), for periods of either 48 or 96 hours. During this time the mice of both groups continued to eat the same thiouracil-containing diet, and the hyperthyroid group was supplemented, in addition, with thyroid, as described above. The excreta were collected during the entire experimental period. The mice were then sacrificed.

Chemical Procedures—The carcass and excreta were hydrolyzed in 30 per cent weight per volume KOH solution in 25 per cent volume per volume alcohol. The solid residues remaining after hydrolysis of the feces were re-extracted for 40 hours in a Soxhlet apparatus, first with 95 per cent ethanol and then with diethyl ether; these extracts were combined with the fecal hydrolysates.

The alkaline solutions were then extracted with diethyl ether in separatory funnels until no further radioactivity could be detected in the ether extract. The ether extracts were combined and washed thoroughly with distilled water; the washings were added to the alkaline hydrolysates. To the ether solutions which contained the non-saponifiable fraction, a solution containing 1.0 per cent (weight per volume) digitonin in 80 per cent (volume per volume) alcohol was added in order to precipitate cholesterol. The sterol digitonide was sedimented by centrifugation, washed three times with alcohol-acetone (1:1) and three times with ether, and then dried to constant weight. Although it was realized that this precipitate very likely contained small amounts of other sterol digitonides (6), no further purification was attempted, and this material was designated the sterol fraction.

The remaining alkaline hydrolysates were placed in an ice bath, adjusted to pH 1 with concentrated H_2SO_4, and extracted with ether. This fraction was designated the saponifiable fraction. Samples were taken and evaporated to constant weight in tared aluminum dishes.

Determination of Radioactivity—All sterol digitonide samples were converted to $BaCO_3$ by combustion. These samples were then counted in aluminum planchets, with a gas flow counter, for a sufficient length of time so that the probable error would not exceed 3 per cent. Observed activities were corrected for self-absorption (7). The saponifiable material was plated directly on aluminum planchets, fitted with lens paper, and the counts obtained were converted to counts as $BaCO_3$ by a predetermined correction factor (8).

[4] Thyroid powder, U. S. P., Armour and Company, Chicago.

Results

Animals receiving thyroid showed symptoms typical of hyperthyroidism; *e.g.*, physical activity, excitability, rate of respiration, water and food consumption, and fecal excretion were significantly increased.

Fig. 1 shows the distribution of carbon 4 of cholesterol in the sterol and saponifiable fractions of carcass and excreta of hyper- and hypothyroid

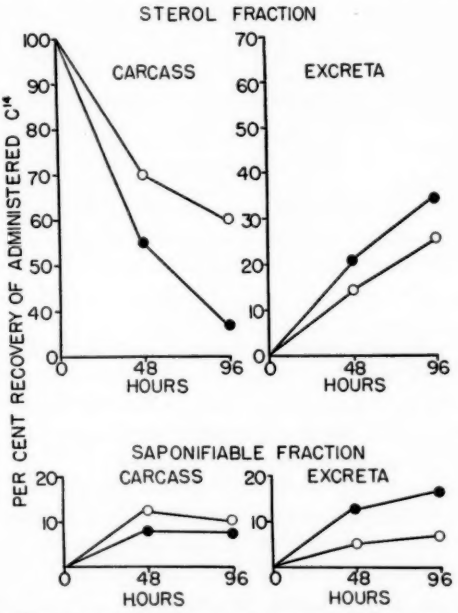


FIG. 1. The distribution of C^{14} in sterol and saponifiable fractions of carcass and excreta of hyper- (●) and hypothyroid (○) mice, 48 and 96 hours after intravenous administration of 4-C^{14}-cholesterol.

mice 48 and 96 hours following the administration of radioactive cholesterol. The statistical significance of these results may be evaluated from Table I, which presents the data for the 96 hour period only, in order to save space.

Fig. 1 and Table I indicate that the labeled carcass cholesterol decreased with time in both groups. The rate of disappearance, however, was significantly greater in the thyroid-fed mice. The hyperthyroid animals excreted the radioactive sterol also more rapidly, but the difference between the fecal values of the two groups was less than that observed for the carcass. In either group, only a fraction of the loss in labeled carcass sterol was accounted for as fecal sterol. The saponifiable fraction of both groups

contained significant quantities of radioactivity; however, the hyperthyroid mice excreted about 2.7 times as much labeled saponifiable material as the hypothyroid group.

TABLE I

Per Cent Distribution of C^{14} in Hyper- and Hypothyroid Mice 96 Hours after Intravenous Administration of 4-C^{14}-Cholesterol

Endocrine supplements	No. of mice	Mean recovery of administered C^{14}						Total counts recovered
		Sterol fraction			Saponifiable fraction			
		Carcass	Excreta	Total	Carcass	Excreta	Total	
Thyroid + thiouracil	5	36 ± 2	34 ± 2	70 ± 3	8 ± 1	16 ± 2	24 ± 1	94 ± 2
Thiouracil	4	61 ± 3	26 ± 2	87 ± 1	10 ± 2	6 ± 1	16 ± 2	103 ± 1
P for difference*		0.001	0.02	0.001	0.4	0.01	0.01	

* The *P* values were calculated from Fisher's table of *t*.

DISCUSSION

The results were interpreted to indicate that thyroid can stimulate, under ordinary nutritional conditions, the conversion of cholesterol to acid products as well as the excretion of the sterol and of some of the products formed. These findings confirm and extend earlier observations on enhanced cholesterol destruction in the hyperthyroid state (1, 2, 9). However, these reports were based on different experimental approaches, such as balance studies, in which non-radioactive cholesterol (1, 9) was employed, and turnover experiments dealing with the incorporation of tritium into cholesterol (2), and the nature of the products formed was not indicated.

Friedman *et al.* observed that the rate of cholesterol excretion was increased in the hyperthyroid rat (10). These investigators postulated that this phenomenon could be a result of augmented cholesterol synthesis, primarily in the liver. The studies reported here indicate that the thyroid hormone may enhance cholesterol excretion by stimulating mobilization of tissue sterol as well. On the other hand, Mosbach *et al.* concluded from experiments on normal and hypothyroid dogs that the thyroid state had only a minor effect on the excretion of sterols and bile acids (11).

It has been established that thyroid hormone depresses plasma and liver cholesterol (12, 13). The results described above indicate that the hormone may accomplish this effect by means of enhancing both the destruction and excretion of the sterol, in agreement with considerations by Rosenman *et al.* (2).

The data reported for the saponifiable fraction show that the thyroid hormone stimulates the conversion of cholesterol to acid products. This

acid material may include bile acids, as the formation of cholic acid from cholesterol was demonstrated (14). The possibility has to be considered, therefore, that thyroid stimulates the conversion of cholesterol to bile acids. On the other hand, Thompson and Vars reported that thyroid suppresses cholic acid excretion in rats whose common bile duct had been cannulated (15), and the formation of fecal acidic products, other than bile acids, from cholesterol was also demonstrated (16).

The difference in the total recovery of radioactivity between the two experimental groups was too small to merit further consideration, although the possibility cannot be excluded that the thyroid hormone stimulates also the conversion of cholesterol to products different from those recovered in the sterol and saponifiable fractions.

SUMMARY

Hyper- and hypothyroid mice were injected with radioactive cholesterol. The distribution of isotope in the sterol and saponifiable fractions of carcass and excreta was determined after periods of 48 and 96 hours.

It was observed that the thyroid hormone stimulated (1) the degradation of cholesterol, in particular, its conversion to acidic products, and (2) the excretion of the sterol itself and of some of the acidic products formed.

The authors would like to thank Dr. Irving Zabin of the University of California at Los Angeles for his interest and helpful advice.

BIBLIOGRAPHY

1. Weiss, S. B., Henkin, G., and Marx, W., *Proc. Soc. Exp. Biol. and Med.*, **86,** 800 (1954).
2. Rosenman, R. H., Byers, S. O., and Friedman, M., *J. Clin. Endocrinol. and Metabolism*, **12,** 1287 (1952).
3. Schwenk, E., Gut, M., and Belisle, J., *Arch. Biochem. and Biophys.*, **31,** 456 (1951).
4. Meier, J. R., Siperstein, M. D., and Chaikoff, I. L., *J. Biol. Chem.*, **198,** 105 (1952).
5. Wainfan, E., Henkin, G., Rice, L. I., and Marx, W., *Arch. Biochem. and Biophys.*, **38,** 187 (1952).
6. Frantz, I. D., Jr., and Bucher, N. L. R., *J. Biol. Chem.*, **206,** 471 (1954).
7. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. E., Isotopic carbon, New York (1949).
8. Entenman, C., Lerner, S. R., Chaikoff, I. L., and Dauben, W. G., *Proc. Soc. Exp. Biol. and Med.*, **70,** 364 (1949).
9. Hurxthal, L. M., and Perkin, H. J., *Lahey Clin. Bull.*, **1,** 19 (1938).
10. Friedman, M., Byers, S. O., and Rosenman, R. H., *Circulation*, **5,** 657 (1952).
11. Mosbach, E. H., Abell, L. L., and Fendall, F. E., *Tr. New York Acad. Sc.*, **16,** 156 (1954).
12. Turner, K. B., Present, C. H., and Bidwell, E. H., *J. Exp. Med.*, **67,** 111 (1938).
13. Handler, P., *J. Biol. Chem.*, **173,** 295 (1948).
14. Bloch, K., Berg, B. N., and Rittenberg, D., *J. Biol. Chem.*, **149,** 511 (1943).
15. Thompson, J. C., and Vars, H. M., *Proc. Soc. Exp. Biol. and Med.*, **83,** 246 (1953).
16. Cook, R. P., Polgar, N., and Thomson, R. O., *Biochem. J.*, **47,** 600 (1950).

(*From*

Th
monl
were
ies w
taine
the p

M
ploye
In
unlal
resus
of ab
were
μc. o
mg.
pensi
ganis
orgar
petit
dium

* P
Wash
† I
[1] A
and c
Wash
[2] S
[3] T
4.5 w
[4] T
millia
ploye
used

XUM

VMINO ACID BIOSYNTHESIS IN TORULOPSIS UTILIS AND NEUROSPORA CRASSA

By PHILIP H. ABELSON* and HENRY J. VOGEL†

(From the Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D. C., and the Department of Microbiology, Yale University, New Haven, Connecticut)

(Received for publication, July 12, 1954)

The present investigation is concerned with the biosynthesis of the commonly occurring open chain amino acids and proline. The methods used were tracer techniques, including isotopic competition. Since similar studies were recently carried out with *Escherichia coli* (1–3),[1] the results obtained here with *Torulopsis utilis* and *Neurospora crassa* are discussed from the point of view of comparative biochemistry.

EXPERIMENTAL

Methods—For the isotopic competition experiments, the methods employed were analogous to those described for *E. coli* (1).[2]

In the case of *T. utilis* (ATCC 9950), cultures growing vigorously in unlabeled glucose-salt medium[3] were centrifuged, and the organisms were resuspended in fresh glucose-free salt medium to give an optical density of about 0.075 at 650 mμ. To 20 ml. portions of the resulting suspensions were added supplements of 5 mg. of unlabeled glucose, about 5 to 10 μc. of a C^{14}-labeled tracer[4] (glucose or other tracer as indicated), and 2 mg. of any of the desired unlabeled competitors. The supplemented suspensions were incubated at about 25° with aeration until the mass of organisms approximately doubled. In some instances (as indicated), the organisms were not resuspended in fresh medium, but the tracers and competitors were added simultaneously to cultures growing in unlabeled medium.

* Present address, Geophysical Laboratory, Carnegie Institution of Washington, Washington, D. C.

† Damon Runyon Memorial Fellow.

[1] A comprehensive report on tracer studies of amino acid biosynthesis in *E. coli* and other microorganisms is to be published shortly as a Carnegie Institution of Washington monograph.

[2] See this reference for citations of earlier work.

[3] The medium was similar to that of Baddiley *et al.* (4); an initial pH of about 4.5 was employed, and glucose (1 per cent) was substituted for sucrose.

[4] The specific activity of the uniformly labeled tracers used was about 6 mc. per milliatom of carbon; that of the glutamic acid-1-C^{14} and of the acetate-1-C^{14} employed was about 1 mc. per mmole. Unless otherwise indicated, the C^{14} compounds used were uniformly labeled.

In the case of *N. crassa* (ATCC 10336), a similar procedure was followed, except for modifications necessitated by the cultural habits of this organism. An appropriate medium[5] and strong agitation (to insure a dispersed condition of the culture) were employed. About 1 mg. (dry weight) of freshly harvested *N. crassa* was used per 20 ml. of the supplemented mixtures.

In addition to the isotopic competition studies, tracer incorporation experiments were carried out in which unlabeled glucose and a given C^{14} tracer were the only supplements.[6]

After cultivation, the organisms were harvested and subjected to the fractionation, paper chromatography, and radioautography procedures previously described (1). Isotopic competition was determined as before (1). In the tracer incorporation experiments the relative specific activities of various protein amino acids were determined (see Abelson (1)).

Materials—The C^{14}-carbon dioxide used was generated from barium C^{14}-carbonate obtained from the Oak Ridge National Laboratory. Uniformly labeled C^{14}-glucose was prepared from canna leaves (6, 7) which had assimilated C^{14}-carbon dioxide. Uniformly labeled L-amino acids (except ornithine) were isolated from *Chlorella pyrenoidosa*, which had been grown on C^{14}-carbon dioxide as a carbon source (8). Labeled L-ornithine was prepared from uniformly C^{14}-labeled L-arginine by the action of arginase (9). L-Glutamic acid-1-C^{14} was isolated from *E. coli* cultivated in the presence of C^{14}-carbon dioxide (10). Sodium acetate-1-C^{14} was obtained from a commercial source. The unlabeled L-amino acids used were commercial products. Several of the keto acids, including *d*-α-keto-β-methylvaleric acid (the keto analogue of L-isoleucine), were kindly furnished by Dr. Alton Meister. Synthetic N^{α}-acetyl-L-ornithine (11) and DL-glutamic γ-semialdehyde (12), which is in equilibrium with the cyclized Δ^1-pyrroline-5-carboxylic acid, were prepared as previously described.

RESULTS AND DISCUSSION

Classification of Amino Acids—The present results obtained with *T. utilis* and *N. crassa* support the subdivision of the open chain amino acids and proline into five groups on the basis of the biosynthetic origin of their carbon skeletons. As illustrated in Table I for *T. utilis* and *N. crassa*, with aspartate as tracer, the protein amino acids shown fall into three distinct groups with respect to relative specific activity. One group con-

[5] A modification of the medium of Beadle and Tatum (5) was employed. Tartrate was omitted to avoid possible complications, although it is not a carbon source for *N. crassa* (5). The modified medium supported adequate growth for the present purposes.

[6] The differentiation between competition and incorporation is made to emphasize differences in experimental design and for purposes of exposition.

sists of glutamate, proline, and arginine (glutamic family), a second of aspartate, methionine, threonine, and isoleucine (aspartic family), and the third of lysine. The results with carbon dioxide, acetate, or glutamate as tracer are in accord with this classification.

As shown in Table II, with alanine as tracer, heavy incorporation into protein alanine, valine, and leucine (pyruvic family) was observed, along

TABLE I

*Incorporation of Tracers into Protein Amino Acids of T. utilis and N. crassa (As Relative Specific Activity)**

Tracer	Glu	Arg	Pro	Asp	Thr	Iso	Met	Lys
T. utilis								
C^{14}-Carbon dioxide†	100	500	95	230	230	240	220	0
Acetate-1-C^{14}†	100	250	129	33	30	30	35	200
C^{14}-Aspartate	100	100	105	167	160	170	170	70
C^{14}-Glutamate	100	80	67	26	25	24	22	40
Glutamate-1-C^{14}	100	90	70	5	0	0	0	0
N. crassa								
C^{14}-Carbon dioxide†	100	440	100	160	160	160	160	0
Acetate-1-C^{14}	100	85	90	90	90	90		93
C^{14}-Aspartate	100	85	100	250	250	250	250	50
C^{14}-Glutamate	100	70	90	51	49	48		47
Glutamate-1-C^{14}	100	60	80	5	0	0		0

* The specific activity figures shown are relative to the respective protein glutamic acid values. See the text for details and foot-note 4. Glu, glutamic acid; Arg, arginine; Pro, proline; Asp, aspartic acid; Thr, threonine; Iso, isoleucine; Met, methionine; Lys, lysine.

† The high relative specific activity of arginine obtained with this tracer is presumably due to incorporation of C^{14} into the amidine carbon atom.

with light incorporation into certain amino acids. The labeling of valine and leucine presumably is introduced via pyruvate. With serine as tracer, approximately equal relative specific activities are obtained in protein serine, glycine, and cysteine (serine family).

Of the remaining protein amino acids, histidine is in a group by itself, as indicated by biosynthetic studies with *N. crassa* (13) and a yeast (14), and the aromatic amino acids form another group in these organisms (15, 16). Thus, the fungal protein amino acids may be classified as follows: (*a*) glutamic acid, proline, arginine; (*b*) aspartic acid, methionine, threonine, isoleucine; (*c*) lysine; (*d*) alanine, valine, leucine; (*e*) serine, glycine,

cysteine (cystine); (*f*) histidine; and (*g*) phenylalanine, tyrosine, tryptophan.

A similar subdivision applies to *E. coli*, except for lysine which in this organism is derived from aspartic acid, as is α,ε-diaminopimelic acid (1, 2). The latter amino acid has not been detected in fungi (17).

The results shown in Table I are in accord with the existence of a Krebs cycle in *T. utilis* and *N. crassa*.

Glutamic Family—The data in Table I are consistent with the conclusion that in *T. utilis* and *N. crassa* the 5-carbon skeletons of proline and arginine

TABLE II

*Incorporation of Tracers into Protein Amino Acids of T. utilis and N. crassa (As Relative Specific Activity)**

C^{14} tracer	Ala	Val	Leu	Ser	Gly	Cys
			T. utilis			
α-Alanine	100	40	60	5	5	
Serine	0	0	0	100	100	100
Glycine	0	0	0	15	100	15
			N. crassa			
α-Alanine	100	63	60	10	10	10
Serine	0	0	0	100	100	100

* With tracer α-alanine, serine, and glycine, the specific activity figures shown are relative to the respective protein α-alanine, serine, and glycine values. See the text for details. Ala, α-alanine; Val, valine; Leu, leucine; Ser, serine; Gly, glycine; Cys, cysteine.

are derived from glutamate. Table III summarizes the results of isotopic competition experiments with *T. utilis*, members of the glutamic family and presumable intermediates being used as competitors. Analogous experiments with *N. crassa* gave qualitatively very similar results. Ornithine, citrulline, and arginine are seen to compete effectively as sources of protein arginine and of protein proline.[7] Proline as competitor is seen to suppress the radioactivity of protein proline but not that of glutamate and arginine. This result does not support the possible major rôle of proline

[7] Added ornithine appears to contribute to the amidine carbon of arginine, possibly via carbon dioxide liberated through decarboxylation. Added ornithine also contributes to glutamate, probably via glutamic γ-semialdehyde. Citrulline and arginine presumably contribute to proline via ornithine, formed through the "ornithine cycle" (18); the ornithine formed may then yield proline via glutamic γ-semialdehyde.

as ornithine precursor proposed for *T. utilis* (19) and *N. crassa* (20). Glutamic γ-semialdehyde contributes to protein proline and to some extent to glutamate and arginine.

Evidence has been obtained with *N. crassa* mutants that glutamic γ-semialdehyde is a precursor not only of proline but also of ornithine and hence arginine (21).[2] The present finding that the semialdehyde contributes to arginine is consistent with a rôle of the semialdehyde as ornithine precursor in *T. utilis* as well as *N. crassa*. However, in view of the contribution of added semialdehyde to glutamate, it seems possible that at least part of the exogenous semialdehyde did not directly form ornithine.

TABLE III

*Effect of Competitors on Radioactivity of Amino Acids of Glutamic Family in T. utilis (As Per Cent Radioactivity of Protein Amino Acids Relative to Control)**

C^{12} competitor	Glu	Arg	Pro
None (control)	100	100	100
Ornithine†	75	12	4
Citrulline†	95	25	25
Arginine†	95	15	15
Glutamic γ-semialdehyde	65	70	6
Proline	100	100	0

* The tracer employed was C^{14}-glucose. See the text for details. Glu, glutamic acid; Arg, arginine; Pro, proline.

† The tracer and competitor were added simultaneously to a culture growing in unlabeled medium.

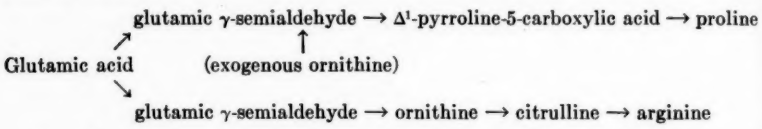
In contrast to the results with the fungi, the semialdehyde does not compete with glutamate and arginine in *E. coli* under analogous conditions (1).

Studies with *N. crassa* mutants have also shown that exogenous, but not *endogenous*, ornithine is a major source of proline, and that glutamic γ-semialdehyde as proline precursor may be distinct from the semialdehyde as ornithine precursor (21). These results have been interpreted as reflecting a physical separation of the proline and ornithine paths (21). This separation was ascribed to a spatial organization of relevant enzyme systems[8] with a resulting more or less restrictive "channeling" of metabolites (21).

[8] Thus the enzyme system involved in the formation of glutamic γ-semialdehyde as proline precursor may differ, with respect to organization and possibly to other properties, from that thought to be involved in the formation of the semialdehyde as ornithine precursor. Also, the enzyme catalyzing the ready conversion of exogenous ornithine to glutamic γ-semialdehyde may differ, as to organization and perhaps as to other properties, from the enzyme thought to be involved in the biosynthetic conversion of glutamic γ-semialdehyde to ornithine. Enzyme organization resulting

The present tracer studies point to a distinction between endogenous and exogenous ornithine in *T. utilis* also. With ornithine (at a concentration of about 1 γ per ml.) as tracer, the specific activities of proline and arginine were in the ratio, 100:6, respectively; when the labeled ornithine was diluted with carrier and used at a concentration of 50 γ per ml., this ratio was 100:80, respectively. These results indicate that the exogenous ornithine as a source of proline does not equilibrate with the endogenous ornithine as a source of arginine. On the other hand, the separation of endogenous from exogenous ornithine is not complete, as illustrated especially by the result with the diluted tracer ornithine.[9]

The present results relating to the glutamic family support the accompanying scheme.[10]


glutamic γ-semialdehyde → Δ¹-pyrroline-5-carboxylic acid → proline
Glutamic acid
(exogenous ornithine)
glutamic γ-semialdehyde → ornithine → citrulline → arginine

The glutamate-proline-ornithine interrelation shown above differs from the one found in *E. coli* in which ornithine is formed via several acetylated intermediates (23, 24).[11] In isotopic competition experiments with *T. utilis* and *N. crassa*, N^{α}-acetylornithine, a precursor of ornithine in *E. coli* (11, 23), did not act as a source of arginine. This finding is in harmony with the failure of N^{α}-acetylornithine to satisfy the growth requirement of *N. crassa* mutants which respond to ornithine (23), and with the lack of detectable acetylornithinase activity in extracts of wild type *N. crassa* (21).

Aspartic Family—Isotopic competition results with members of the as-

---

in "channeling" may be expected to promote ornithine synthesis via glutamic γ-semialdehyde by counteracting the tendency of the semialdehyde to cyclize, or by facilitating the removal of ornithine formed, or by both.

[9] Furthermore there may be a distinction between endogenous and exogenous glutamate in both *T. utilis* and *N. crassa*, as suggested by the results given in Table I. When the glutamic family is labeled via endogenous glutamate, *e.g.* with aspartate as tracer, the specific activities of the members of this family are generally less variable than with exogenous glutamate as tracer. The relative specific activities of the members of the glutamic family, moreover, vary with cultural conditions, such as washing of the cells in the case of *T. utilis*. In this organism, as well as in *N. crassa*, the extent of labeling of the glutamic family may well be affected by the nature and size of possible glutamate reservoirs (22).

[10] Glutamic γ-semialdehyde is shown twice to emphasize the possibility that glutamic γ-semialdehyde as proline precursor and glutamic γ-semialdehyde as ornithine precursor do not form a common pool. The quantitative significance of the step from glutamic γ-semialdehyde to ornithine has not been ascertained.

[11] In *E. coli*, a minor path connects the main routes to ornithine and proline (24).

partic family, and intermediates are given in Table IV for *N. crassa*. Very similar competition results were obtained with *T. utilis* with C^{14}-carbon dioxide as tracer. In addition, α-aminobutyrate was found to contribute

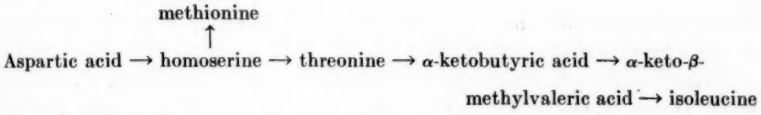
TABLE IV

*Effect of Competitors on Radioactivity of Amino Acids of Aspartic and Pyruvic Families in N. crassa (As Per Cent Radioactivity of Protein Amino Acids Relative to Control)**

C^{14} tracer	C^{12} competitor	Asp	Thr	Iso	Met	Ala	Val	Leu
Carbonate	None (control)	100	100	100	100			
"	Homoserine	100	7	7	6			
"	Threonine	90	8	6				
"	Isoleucine	100	100	12				
Glucose	None (control)	100	100	100		100	100	100
"	α-Ketobutyrate	100	100	35				
"	α-Keto-β-methylvalerate	100	100	5				
"	Pyruvate					75	63	58
"	α-Ketoisovalerate					100	64	55
"	α-Ketoisocaproate					100	100	25
"	Valine					100	43	62
"	Leucine					100	100	10

* See the text for details. Asp, aspartic acid; Thr, threonine; Iso, isoleucine; Met, methionine; Ala, α-alanine; Val, valine; Leu, leucine.

to protein isoleucine in both organisms. These results support the paths in the accompanying diagram.

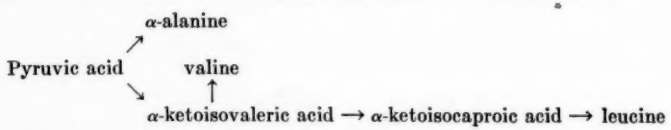


The function of aspartate as threonine precursor has previously been shown for a yeast (25), but apparently not for *N. crassa*. In the latter organism homoserine has been found to yield threonine and methionine (26), and α,β-dihydroxy-β-methylvalerate (27) forms isoleucine via its α-keto analogue (28). In *E. coli* similar interrelations among metabolites derived from aspartate have been found, except that in this organism lysine and α,ε-diaminopimelic acid are also aspartate derivatives (2).

Lysine—The data in Table I are consistent with the view that in *T. utilis* the carbon chain of lysine is built up through a combination of the succinate moiety of α-ketoglutarate with acetate (or a related compound), in confirmation of earlier results (29, 30). The results in Table I indicate

that *N. crassa* also utilizes the succinate moiety of α-ketoglutarate for lysine synthesis, but (in contrast to *T. utilis*) derives the remaining 2 carbon atoms of lysine from a precursor which does not appear to equilibrate with exogenous acetate or with Krebs cycle acetate.[12] In neither fungus does aspartate serve as a major lysine precursor, as it does in *E. coli*.

Pyruvic Family—The incorporation data in Table II with alanine as tracer show labeling of alanine, valine, and leucine. As mentioned above, the labeling of valine and leucine presumably is introduced via pyruvate. In Table IV isotopic competition data for the pyruvic family in *N. crassa* are presented. Closely similar results were obtained in analogous experiments with *T. utilis*.[13] These data support the scheme shown below.



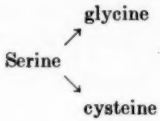
The rôle of pyruvate as valine precursor has previously been shown in *E. coli* (1) and in *T. utilis* (31). In *N. crassa*, α,β-dihydroxyisovalerate (32) and α-ketoisovalerate (28) are known to yield valine. The function of α-ketoisovalerate as a precursor of α-ketoisocaproate and leucine has thus far been demonstrated in *E. coli* only (1). The present results show that the two fungi synthesize leucine in the same manner as does *E. coli*. The conversion of α-ketoisovalerate to α-ketoisocaproate is suggested to involve the loss of a 1-carbon fragment (presumably the carboxyl carbon of α-ketoisovalerate) and addition of a 2-carbon fragment which is probably derived from acetate or a related compound, in view of the finding by Ehrensvärd *et al.* (29) that in *T. utilis* the carboxyl group of leucine appears to come from acetate carboxyl.

Serine Family—As illustrated in Table II for both *T. utilis* and *N. crassa*, tracer serine labels protein serine, glycine, and cysteine at equal specific activity. However, with tracer glycine in *T. utilis*, serine and cysteine were found to have only 15 per cent each of the specific activity of protein glycine.

These results indicate the following metabolic relationship among members of the serine family.

[12] Thus, in *N. crassa*, the results with C^{14}-glutamate as tracer are consistent with a contribution of 4 carbon atoms to lysine via α-ketoglutarate; with glutamate-1-C^{14} as tracer, lysine does not become labeled; and with acetate-1-C^{14} as tracer, the specific activity of the lysine is equal to only about that of the protein glutamate. In contrast, in *T. utilis*, with acetate-1-C^{14} as tracer the specific activity of lysine is twice that of protein glutamate.

[13] In incorporation experiments with *T. utilis* and *N. crassa*, with C^{14}-valine as tracer, significant labeling was observed only in protein valine and leucine.



It has previously been concluded (*e.g.* by Ehrensvärd *et al.* (33)) that glycine can form serine in yeast. Apparently, under the cultural conditions used here, serine is a major precursor of both glycine and cysteine in the two fungi. The same relationship has been observed in *E. coli* (1).[14]

There are, however, at least three differences in serine metabolism between *E. coli* (1) and the two fungi when these organisms are grown on glucose-salt medium. In *E. coli*, but not in the fungi, exogenous threonine contributes to serine, and serine contributes to members of the pyruvic family; in *T. utilis*, but not in *E. coli*, glycine can yield serine and cysteine, as shown in Table II.

SUMMARY

The biosynthesis of open chain amino acids and proline in *Torulopsis utilis* and *Neurospora crassa* was studied by means of tracer techniques. The results are in harmony with the existence of a Krebs cycle in these organisms; they permit the classification of the amino acids studied into groups on the basis of biosynthetic origin, and they support the following biosynthetic sequences (in both *T. utilis* and *N. crassa*): (*a*) glutamic acid, glutamic γ-semialdehyde, Δ^1-pyrroline-5-carboxylic acid, proline; (*b*) glutamic acid, glutamic γ-semialdehyde, ornithine, citrulline, arginine; (*c*) aspartic acid, homoserine, threonine, α-ketobutyric acid, α-keto-β-methylvaleric acid, isoleucine; (*d*) homoserine, methionine; (*e*) α-ketoglutaric acid, lysine; (*f*) pyruvic acid, α-alanine; (*g*) pyruvic acid, α-ketoisovaleric acid, α-ketoisocaproic acid, leucine; (*h*) α-ketoisovaleric acid, valine; (*i*) serine, glycine; (*j*) serine, cysteine. *T. utilis* and *N. crassa* apparently differ from each other in details of lysine formation. Both organisms differ from *E. coli* in several respects, including the glutamate-proline-ornithine relationship, lysine synthesis, as well as aspartate and serine metabolism.

BIBLIOGRAPHY

1. Abelson, P. H., *J. Biol. Chem.*, **206**, 335 (1954).
2. Abelson, P. H., Bolton, E., Britten, R., Cowie, D. B., and Roberts, R. B., *Proc. Nat. Acad. Sc.*, **39**, 1020 (1953).
3. McQuillen, K., and Roberts, R. B., *J. Biol. Chem.*, **207**, 81 (1954).
4. Baddiley, J., Ehrensvärd, G., Johansson, R., Reio, L., Saluste, E., and Stjernholm, R., *J. Biol. Chem.*, **183**, 771 (1950).

[14] The serine-glycine relationship in serine-requiring mutants of *E. coli* has been investigated by J. O. Meinhart and S. Simmonds (personal communication).

5. Beadle, G. W., and Tatum, E. L., *Proc. Nat. Acad. Sc.*, **27,** 499 (1941).
6. Cederfriend, S., and Gibbs, M., *Science*, **110,** 708 (1949).
7. Putman, E. W., and Hassid, W. Z., *J. Biol. Chem.*, **196,** 749 (1952).
8. Abelson, P. H., Bolton, E. T., Britten, R. J., Cowie, D. B., Roberts, R. B., and Lövtrup, S., in *Carnegie Institution of Washington Yr. Bk.*, No. 51, 87 (1952).
9. Hunter, A., and Downs, C. E., *J. Biol. Chem.*, **155,** 173 (1944).
10. Abelson, P. H., Bolton, E. T., and Aldous, E., *J. Biol. Chem.*, **198,** 165 (1952).
11. Vogel, H. J., Abstracts, American Chemical Society, 122nd meeting, Atlantic City, 43C (1952).
12. Vogel, H. J., and Davis, B. D., *J. Am. Chem. Soc.*, **74,** 109 (1952).
13. Ames, B. N., Mitchell, H. K., and Mitchell, M. B., *J. Am. Chem. Soc.*, **75,** 1015 (1953).
14. Levy, L., and Coon, M. J., *J. Biol. Chem.*, **208,** 691 (1954).
15. Tatum, E. L., and Perkins, D. D., *Ann. Rev. Microbiol.*, **4,** 129 (1950).
16. Gilvarg, C., and Bloch, K., *J. Biol. Chem.*, **199,** 689 (1952).
17. Work, E., and Dewey, D. L., *Abstr., 6th Internat. Cong. Microbiol.*, Rome, 33 (1953).
18. Srb, A. M., and Horowitz, N. H., *J. Biol. Chem.*, **154,** 129 (1944).
19. Strassman, M., and Weinhouse, S., *J. Am. Chem. Soc.*, **74,** 1726 (1952).
20. Fincham, J. R. S., *Biochem. J.*, **53,** 313 (1953).
21. Vogel, H. J., and Bonner, D. M., *Proc. Nat. Acad. Sc.*, **40,** 688 (1954).
22. Halvorson, H. O., and Spiegelman, S., *J. Bact.*, **65,** 496 (1953).
23. Vogel, H. J., *Proc. Nat. Acad. Sc.*, **39,** 578 (1953).
24. Vogel, H. J., Abelson, P. H., and Bolton, E. T., *Biochim. et biophys. acta*, **11,** 584 (1953).
25. Black, S., and Wright, N. G., *Federation Proc.*, **13,** 184 (1954).
26. Teas, H. J., Horowitz, N. H., and Fling, M., *J. Biol. Chem.*, **172,** 651 (1948).
27. Adelberg, E. A., Bonner, D. M., and Tatum, E. L., *J. Biol. Chem.*, **190,** 837 (1951).
28. Bonner, D., Tatum, E. L., and Beadle, G. W., *Arch. Biochem.*, **3,** 71 (1943–44).
29. Ehrensvärd, G., Reio, L., Saluste, E., and Stjernholm, R., *J. Biol. Chem.*, **189,** 93 (1951).
30. Strassman, M., and Weinhouse, S., *J. Am. Chem. Soc.*, **75,** 1680 (1953).
31. Strassman, M., Thomas, A. J., and Weinhouse, S., *J. Am. Chem. Soc.*, **75,** 5135 (1953).
32. Adelberg, E. A., and Tatum, E. L., *Arch. Biochem.*, **29,** 235 (1950).
33. Ehrensvärd, G., Sperber, E., Saluste, E., Reio, L., and Stjernholm, R., *J. Biol. Chem.*, **169,** 759 (1947).

CONVERSION OF PYRUVIC ACID-2-C^{14} TO SOME ALIPHATIC MONOAMINO ACIDS IN YEAST*

By CHIH H. WANG, BERT E. CHRISTENSEN, and VERNON H. CHELDELIN

(From the Department of Chemistry and the Science Research Institute, Oregon State College, Corvallis, Oregon)

(Received for publication, July 26, 1954)

Previous papers from this laboratory (1–5) have reported the utilization of pyruvate-2-C^{14} by bakers' yeast under aerobic and anaerobic conditions, as well as the isotopic distribution in aspartic acid, glutamic acid, and tyrosine isolated from the yeast hydrolysate. The present communication extends these studies to include several aliphatic amino acids; namely, alanine, threonine, glycine, serine, and valine.

EXPERIMENTAL

Growth of Yeast—Growth of the test organism, *Saccharomyces cerevisiae* (bakers' yeast), was carried out as described in a previous communication (1). Briefly, it consisted of culturing the cells for 16 hours in a malt extract-urea-phosphate medium, transferring the culture for 4 hours into a glucose-salts-vitamins-$(NH_4)_2SO_4$ medium, and finally transferring 4.0 gm. (dry weight) of the cells into 1 liter of a salts-vitamins-$(NH_4)_2SO_4$ medium containing 20 mM $CH_3C^{14}OCOOH$. After 4 to 5 hours incubation at 30° in oxygen or nitrogen, the pyruvate was completely utilized and the yeast mass had increased to 4.4 gm. The pyruvate was synthesized from $CH_3C^{14}OOH$ via pyruvonitrile according to the method of Thomas *et al.* (6). The specific activity was 18.5×10^5 c.p.m. per mmole.

Isolation of Amino Acids—After hydrolysis of the defatted yeast and removal of acidic and basic amino acids by ion exchange columns (2) and tyrosine by crystallization with a non-isotopic carrier, the neutral amino acids were adsorbed on a Dowex 50 column (28×700 mm.) and separated by elution with HCl according to the method of Stein and Moore (7). After removal of HCl with Ag_2CO_3, glycine, alanine, and valine were isolated from the corresponding fractions by means of concentration and ethanol precipitation and purified by recrystallization from aqueous ethanol. Threonine was separated from serine in the fractions containing these two amino acids by means of chromatography on a cellulose powder column (8). The identity and purity of each amino acid were established

* This research was supported by contract No. AT (45-1)-301 from the Atomic Energy Commission. Published with the approval of the Monographs Publications Committee, Research paper No. 258, School of Science, Department of Chemistry.

by paper chromatography, prior to dilution with non-isotopic carriers for degradation studies. The yields were variable, since only the fractions containing single amino acids from the ion exchange columns were recovered. When the yields were small (10 to 50 mg.), carrier was added to a prescribed level of radioactivity, usually 10^3 c.p.m. per mmole of carbon.

Degradation Procedures—Alanine was degraded by (*a*) total combustion for the specific activity of the whole molecule; (*b*) ninhydrin decarboxylation for the specific activity of the carboxyl carbon; (*c*) a portion of the acetaldehyde recovered from the ninhydrin reaction was converted to iodoform, which on combustion gave the specific activity of carbon 3; and (*d*) the remainder of the acetaldehyde was isolated as the 2,4-dinitrophenylhydrazone, which was then converted to CO_2 to give the specific activities of carbons 2 and 3.

Threonine was degraded as follows: (*a*) total combustion for the specific activity of the whole molecule; (*b*) ninhydrin decarboxylation for the specific activity of carbon 1; (*c*) periodate cleavage according to the procedure of Ehrensvärd *et al.* (9) to give acetaldehyde, which was isolated as its 2,4-dinitrophenylhydrazone and gave upon combustion the specific activities of carbons 3 and 4; (*d*) conversion of the acetaldehyde from the periodate reaction to iodoform gave the specific activity of carbon 4; and (*e*) the formic acid produced in the periodate treatment was oxidized to CO_2 (10) for C^{14} in carbon 2 of threonine.

Glycine was degraded by combustion to CO_2 and by ninhydrin decarboxylation. The degradation of serine was carried out according to the method of Sakami (10).

The degradation of valine was accomplished in the following manner: (*a*) total combustion for the specific activity of the whole molecule; (*b*) ninhydrin decarboxylation for the specific activity of the carboxyl carbon; (*c*) nitrous acid deamination, followed by dichromate oxidation of the resulting hydroxy acid, yielded acetone, corresponding to carbon atoms 3, 4, and 4′; (*d*) hypoiodite oxidation of acetone to iodoform yielded carbon atoms 4 and 4′; and (*e*) the specific activities of carbon atoms 2 and 3 were determined by difference.

The dichromate oxidation of valine was carried out according to the method of Block and Bolling (11) and adapted to a 2 mmole scale. The reaction products, acetone (from carbon atoms 3, 4, and 4′) and CO_2 (from carbon atoms 1 and 2), were trapped individually in a series of absorption towers comprising two each of ice water and CO_2-free 0.1 N NaOH. A portion of the aqueous acetone solution was treated with $HgSO_4$ and H_2SO_4 (11), and the precipitate of acetone mercury sulfate was collected for determination of specific activity. The bulk of the acetone solution was used for conversion to iodoform (yielding C-4 and C-4′).

All samples were counted as barium carbonate in the conventional manner, with a thin mica window Geiger-Müller counter; the data were corrected for background and self-absorption.

TABLE I

Distribution of C^{14} in Amino Acids from Yeast Utilizing $CH_3C^{14}OCOOH$

Amino acid	Carbon atom	Radioactivity			
		Aerobic pyruvate		Anaerobic pyruvate	
		c.p.m. × 10^5*	*per cent of total*	*c.p.m.* × 10^5	*per cent of total*
Alanine	Total	2.19	100	2.08	100
	1. COOH	0.51	23	0.14	7
	2. $CHNH_2$	1.46	67	1.88	90
	3. CH_3	0.22	10	0.06	3
Threonine	Total	3.66	100	2.87	100
	1. COOH	0.64	17.5	0.79	27.5
	2. $CHNH_2$	2.22	61	0.72	25
	3. CHOH	0.27	7.5	0.56	19.5
	4. CH_3	0.53	15	0.83	29
Glycine	Total	1.81	100	1.75	100
	1. COOH	0.85	47	0.70	40
	2. CH_2NH_2	0.96	53	1.05	60
Serine	Total	2.84	100	1.38	100
	1. COOH	1.16	41	0.49	42
	2. $CHNH_2$	1.60	57	0.55	48
	3. CH_2OH	0.06	2	0.11	10
Valine	Total	4.36	100	2.98	100
	1. COOH	0.39	9	0.07	3
	2. $CHNH_2$	2.50	57	1.54	52
	3. CH_2	1.17	27	1.35	45
	4, 4′. CH_3, CH_3	0.30	7	0.01	0

* Specific activity (total) is expressed as counts per minute per millimole of amino acid; the activities of individual carbon atoms are counts per minute per millimole of carbon. All values are given in terms of undiluted amino acids.

RESULTS AND DISCUSSION

The specific activities of the various amino acids, as well as the intramolecular distribution of C^{14} in each amino acid, are reported in Table I.

Glycine and Serine—The similar labeling of C-1 and C-2 in these amino acids confirms their well known interconversion. The origin of the pair has been postulated through formation of serine from pyruvate in the rat (12); however, it is evident from Table I that this cannot be true in yeast because of the non-equivalence of serine and alanine; the pathway from pyruvate to serine must be less direct and must involve one or more steps

that permit equilibration of carbons 1 and 2 of serine. The divergent pathways of serine production in yeast and animals are further indicated by the administration of $C^{14}H_3COOH$ to bakers' yeast (Gilvarg and Bloch (13)), wherein this amino acid was labeled as follows: C-3, 81 per cent; C-2, 11 per cent; C-1, 8 per cent. Assuming in these latter experiments that pyruvate equilibrated with oxalacetate, a direct conversion to serine should have provided equal isotope in C-2 and C-3. On the other hand, if glycine *preceded* serine formation, the acetate methyl could have been expected to appear in C-3 of serine and thus produce the preferential labeling observed.

The origin of the glycine skeleton may then be sought from other known metabolites. An α,β cleavage of threonine has been proposed (14); however, this does not appear feasible in yeast, since in the present aerobic sample the isotopic distribution in glycine does not match that in the corresponding atoms of threonine. Also, in a study of $C^{13}H_3C^{14}OOH$ as substrate for *Torula* yeast, Ehrensvärd *et al.* (9) have pointed out that the proposed cleavage of threonine could be expected to produce 2 molecules of acetate, with C^{14} in the methyl group. This was not supported by their findings.

The similar content of C^{14} in C-1 and C-2 of glycine formed from pyruvate-2-C^{14} could result from forward operation of the Krebs cycle, as outlined in Fig. 1, Route A (it will be recalled that C-2 and C-3 of glutamate were equally labeled in this yeast sample (4)). Conversion of α-ketoglutarate to a C_4-dicarboxylic acid, followed by an α,β cleavage, would then produce the isotope pattern observed for glycine. Similar operation of the cycle with acetate-1-C^{14} would provide carboxyl-labeled glycine (Route B), in agreement with the observations of Ehrensvärd *et al.* (9). This scheme (Route A) is also consistent with the observation of Shemin (15) that C-1 of glycine may arise from C-2 of glutamate. An additional pathway, through direct interconversion of C_4 acids formed from pyruvate-2-C^{14} (Route C), would give rise to preferential labeling in glycine-C-2 and serine-C-2. The slightly greater isotope content of the amino carbon in these amino acids (Table I) may reflect a limited contribution from this latter sequence.

Valine—Studies with *Torula* yeast (9) and *Escherichia coli* (16) have indicated that the carboxyl group of valine arises from acetate carboxyl, whereas the remaining carbon atoms are derived from acetate-C-2. From experiments with *E. coli* and *Neurospora* mutants (17, 18) a biosynthetic scheme has been proposed which includes α-hydroxy-β-ketobutyric acid as the key precursor of both valine and isoleucine, probably through head to tail condensation of 2 molecules of acetate (18). More recently the suggestion has been made (19, 20) that, when glucose, lactate, glycine, or

acetate is administered to *Torula* yeast, the biosynthesis of valine involves the formation of acetolactate, followed by migration of the α-methyl group to yield β,β-dimethyl pyruvate.

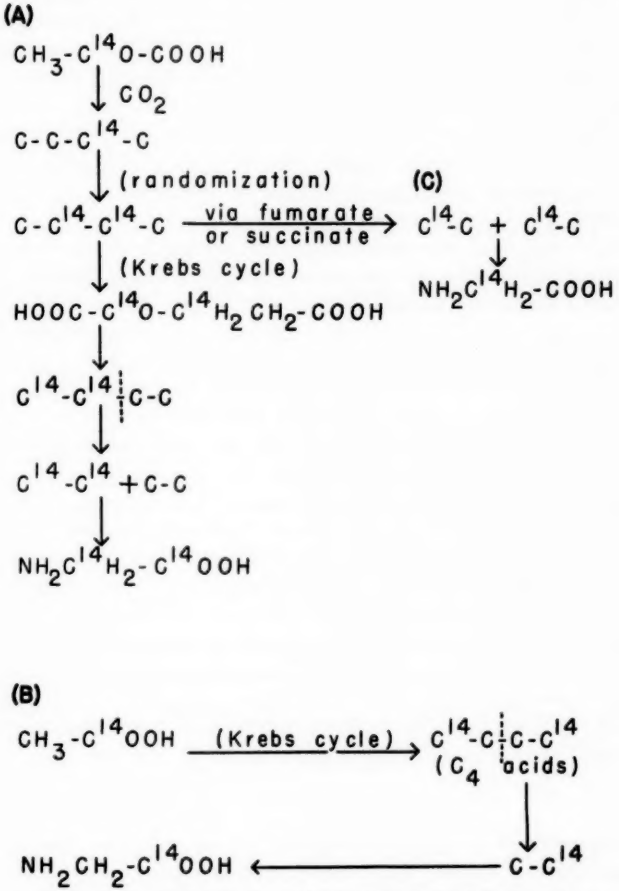


FIG. 1. Suggested routes of formation of glycine in yeast

The isotope pattern in valine (Table I) is similar to that observed by Strassman *et al.* (19), except that in the present work equal labeling in carbons 2 and 3 was not fully realized under aerobic conditions. This could be the result of the non-equivalence of the two condensing partners in the proposed formation of acetolactate (see also McManus (20)). One

other feature of the present work which does not agree completely with the pinacol type rearrangement of acetolactate (19, 20) is the non-equivalence of the C-1:C-2 isotope ratios in valine (0.15 (aerobic) and 0.045 (anaerobic)) and in alanine (0.34 and 0.075). These ratios should be similar if this rearrangement proceeds, assuming that the isotope distribution in alanine may reflect that in pyruvate. It is possible that the differences observed here in carboxyl group labeling in alanine and valine may be due to other reactions, such as reversible decarboxylation in these amino acids; however, preliminary experiments dealing with $C^{14}O_2$ incorporation by this yeast suggest that an additional mechanism may be operative in valine biosynthesis after the formation of acetolactate. This will be discussed in detail elsewhere.

Alanine—As would be expected, alanine appears to be derived directly from the administered pyruvate, presumably by transamination. This is indicated by the heavy labeling in carbon 2 of this amino acid. The minor labeling in carbons 1 and 3 is probably the result of equilibration of pyruvate with oxalacetate. Thus, the activity ratio of C-1:C-3 is 0.51:0.22 = 2.32 for the aerobic sample and 0.14:0.06 = 2.33 for the anaerobic sample, compared to ratios of 2.23 and 2.68 in C-1 and C-3 of aspartic acid. A comparison of the labeling of the carboxyl groups in the aerobic and anaerobic alanine samples indicates that the equilibrium, pyruvate $\rightleftharpoons$ oxalacetate, favors pyruvate under anaerobic conditions.

Threonine—The isotope distribution in threonine samples corresponds closely to that in aspartic acid (3) in both the aerobic and anaerobic yeast. This is in agreement with the close relationship between oxalacetate (or aspartic acid) and threonine in *E. coli* (21) and with the labeling patterns of threonine when $C^{13}H_3C^{14}OOH$ was used as the sole carbon source in yeast and *E. coli* (9, 16). Also, all of these observations are in line with the direct conversion by cell-free yeast preparations (22) of β-aspartyl phosphate to homoserine, as well as with the conclusion that homoserine is converted to threonine in *Neurospora* (23–25) and *E. coli* (26).

SUMMARY

The biosynthesis of glycine, serine, valine, alanine, and threonine has been examined in bakers' yeast, with pyruvate-2-C^{14} as the sole carbon source. In this organism, serine appears to arise from glycine, rather than the reverse, as has been indicated by experiments with rats. Glycine may originate by α,β cleavage of the C_4 acids common to the Krebs cycle. Valine biosynthesis may occur via acetolactate. Alanine appears to be formed directly from pyruvate. The present experiments indicate that the synthesis of threonine proceeds from C_4 acids such as aspartate and homoserine, in support of earlier observations in other organisms.

BIBLIOGRAPHY

1. Wang, C. H., Labbe, R. F., Christensen, B. E., and Cheldelin, V. H., *J. Biol. Chem.*, **197,** 645 (1952).
2. Labbe, R. F., Thomas, R. C., Cheldelin, V. H., Christensen, B. E., and Wang, C. H., *J. Biol. Chem.*, **197,** 655 (1952).
3. Wang, C. H., Thomas, R. C., Cheldelin, V. H., and Christensen, B. E., *J. Biol. Chem.*, **197,** 663 (1952).
4. Wang, C. H., Christensen, B. E., and Cheldelin, V. H., *J. Biol. Chem.*, **201,** 683 (1953).
5. Thomas, R. C., Cheldelin, V. H., Christensen, B. E., and Wang, C. H., *J. Am. Chem. Soc.*, **75,** 5554 (1953).
6. Thomas, R. C., Wang, C. H., and Christensen, B. E., *J. Am. Chem. Soc.*, **73,** 5914 (1951).
7. Stein, W. H., and Moore, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14,** 179 (1949).
8. Blackburn, S., *Chem. and Ind.*, 294 (1951).
9. Ehrensvärd, L., Reio, L., Saluste, E., and Stjernholm, R., *J. Biol. Chem.*, **189,** 93 (1951).
10. Sakami, W., *J. Biol. Chem.*, **187,** 369 (1950).
11. Block, R. J., and Bolling, D., The amino acid composition of proteins and foods, Springfield, 2nd edition (1951).
12. Anker, H. S., *J. Biol. Chem.*, **176,** 1337 (1948).
13. Gilvarg, C., and Bloch, K., *J. Biol. Chem.*, **193,** 339 (1951).
14. Meltzer, H. L., and Sprinson, D. B., *Federation Proc.*, **9,** 204 (1950).
15. Shemin, D., *Cold Spring Harbor Symposia Quant. Biol.*, **14,** 161 (1949).
16. Cutinelli, C., Ehrensvärd, G., Reio, L., Saluste, E., and Stjernholm, R., *Acta chem. Scand.*, **5,** 353 (1951).
17. Umbarger, H. E., and Adelberg, E. A., *J. Biol. Chem.*, **192,** 883 (1951).
18. Tatum, E. L., and Adelberg, E. A., *J. Biol. Chem.*, **190,** 843 (1951).
19. Strassman, M., Thomas, A. J., and Weinhouse, S., *J. Am. Chem. Soc.*, **75,** 5135 (1953).
20. McManus, I. R., *J. Biol. Chem.*, **208,** 639 (1954).
21. Delluva, A. M., *Arch. Biochem. and Biophys.*, **45,** 443 (1953).
22. Black, S., and Wright, N. G., *J. Am. Chem. Soc.*, **75,** 5766 (1953).
23. Teas, H. J., Horowitz, N. H., and Fling, M., *J. Biol. Chem.*, **172,** 651 (1948).
24. Abelson, P. H., Bolton, E. T., and Aldous, E., *J. Biol. Chem.*, **198,** 173 (1952).
25. Cohen, G. N., and Hirsch, M.-L., *J. Bact.*, **67,** 182 (1954).
26. Nisman, B., Cohen, G. N., Wiesendanger, S. B., and Hirsch, M.-L., *Compt. rend. Acad.*, **238,** 1342 (1954).

PARTITION CHROMATOGRAPHY OF HEMINS. SEPARATION OF THE PROSTHETIC GROUPS OF CYTOCHROMES *a* AND a_3*

By MARTIN MORRISON and ELMER STOTZ

(From the Department of Biochemistry, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

(Received for publication, September 29, 1954)

The earlier work of Keilin and Hartree (1) and the later reports of Ball and coworkers (2) and of Chance (3–5) leave little doubt that cytochromes *a* and a_3 are two different hemoproteins. Comparison of the effect of carbon monoxide, as observed spectroscopically at 605 mμ and in the Soret region, indicates pronounced differences in the extinctions of these components and suggests either different hemin prosthetic groups or very different types of linkages between hemin and protein in cytochromes *a* and a_3.

Other workers were concerned with the dichroic ("green") hemin of heart muscle. Thus Negelein (6) separated a hemin which as a reduced pyridine hemochromogen possessed an absorption band at 587 mμ. Roche and Benevent (7) questioned whether this hemin was derived unchanged from the tissue, and the cryptoporphyrin prepared from the hemin was considered by Negelein (8, 9) to be an artifact. More recent work (10–12) leaves no doubt that such a "green" hemin can be derived from tissue and that it is characterized by a reduced pyridine hemochromogen having absorption peaks at 430 and 587 mμ. In addition, Dannenberg and Kiese (13) have isolated a hemin from a partially purified cytochrome oxidase with these absorption characteristics, and Person and coworkers (14) found a similar spectrum upon addition of pyridine to an oxidase preparation. The work cited implies that there is a single prosthetic group (hemin *a*) for cytochromes *a* and a_3.

Falk and Rimington (11), in a thorough investigation of the porphyrins derived from hemin *a*, demonstrated that "porphyrin *a*" was labile to acid and is probably a mixture of at least four porphyrins. Thus porphyrin separation could not lead to valid conclusions as to the possible multiplicity of hemin prosthetic groups, and these investigators were unsuccessful in their attempts to purify hemin *a* by column chromatography. Most recently Kiese and Kurz (15) have described a chromatographic method

* This research was supported in part by grants from the National Heart Institute, National Institutes of Health, United States Public Health Service, and the Life Insurance Medical Research Fund. This work was presented at the meeting of the American Society of Biological Chemists at Chicago, April 6–10, 1953.

XUM

which has separated hemin *a* from protohemin. Only a single hemin *a* fraction was obtained by this method.

It is apparent that a method for separating hemins rather than the porphyrins might be fruitful in supplying information concerning the prosthetic groups of cytochromes *a* and a_3. The object of this paper is to describe a procedure for the partition chromatography of hemins and to demonstrate by this method that cytochromes *a* and a_3 have closely related but different prosthetic groups.

EXPERIMENTAL

Column Chromatography

Preparation of Adsorbent—Silicic acid was the solid adsorbent used to hold the stationary aqueous phase. 500 gm. of reagent grade silicic acid (Merck or Mallinckrodt) are thoroughly mixed with 800 ml. of acid ethanol (400 ml. of c.p. hydrochloric acid diluted to 2400 ml. with 95 per cent ethanol). The mixture is filtered through a Büchner funnel, and the washing procedure is repeated at least three times. The filtrate from the final washing should be colorless. The silicic acid is then washed three times in the same manner with 700 ml. portions of 95 per cent ethanol, and finally three times with 800 ml. portions of diethyl ether. It is then spread on clean white paper and allowed to dry for 24 to 48 hours. The material is then passed through an 80 mesh sieve and dried in a vacuum desiccator.

Chromatography—To 50 gm. of the silicic acid prepared in this way, 20 ml. of 0.15 N hydrochloric acid are added, and the mixture is stirred to a homogeneous powder. To this powder 80 ml. of reagent grade chloroform are added, and the mixture is stirred until a fine slurry is obtained. This slurry is poured into a chromatographic tube 3.5 cm. in diameter and the silicic acid washed in with a minimal amount of chloroform to make a column of approximately 11 cm. in length.

The following procedure is described for the chromatography of hemin solutions which are contaminated with lipides as the result of the tissue extraction procedure. To the column wet with chloroform, 10 ml. of *n*-hexane are added. When 2 to 5 ml. of hexane remain on top of the column, an equivalent volume of a concentrated chloroform solution of the hemins is delivered carefully onto the column (most of the chloroform settles below the hexane layer and precipitation of the hemins is avoided). As the last of the liquid disappears into the column, about 10 ml. of a 1:1 (volume per volume) chloroform-hexane mixture are used to rinse the walls of the tube above the adsorbent. 100 ml. of the chloroform-hexane mixture are then poured onto the column, and, as the last of this solvent passes into the adsorbent, pure chloroform is used to elute the hemins. The use

of hexane accomplishes the removal of lipides from the column prior to the elution of the hemins by pure chloroform.

The steps involving hexane and hexane-chloroform are omitted in the chromatography of lipide-free hemin solutions.

Extraction of Hemins—The starting material for the investigation of the prosthetic groups of cytochromes *a* and a_3 was the purified cytochrome oxidase preparation of Smith and Stotz (16). The precipitate collected after dilution of the Type I oxidase preparation (16) was extracted three

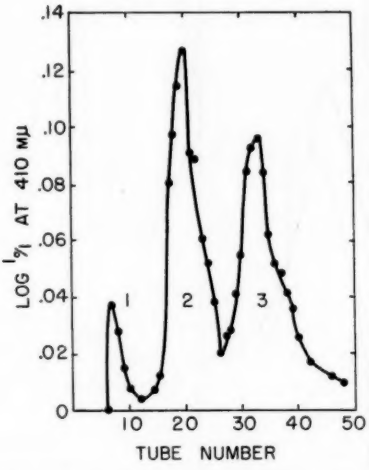


FIG. 1. Chromatograph of hemins extracted from cytochrome oxidase preparation. The hemins represented (Fractions 1, 2, and 3) were prepared from 100 mg. of acetone powder of cytochrome oxidase preparation and chromatographed on a column 1 cm. × 13.5 cm. Each tube for collection contained 3 ml. of eluent.

times with 10 volumes of acetone, then three times with the same volume of chloroform-methanol (2:1 volume per volume), and once again with acetone. These extractions served to remove water and excess lipides. For each gm. of dry residue, 100 ml. of acid acetone (1.8 ml. of concentrated hydrochloric acid per 100 ml. of acetone) are used to extract the hemins. The solid was extracted with stirring at 4° for 15 minutes; this was repeated with fresh solvent until the extract was no longer colored. The combined acid acetone extracts were then diluted with an equal volume of peroxide-free ether in a separatory funnel, and the solution was washed with 10 volumes of 5 per cent sodium chloride solution. The washing procedure was repeated four times. The washed ether solution of hemins was evaporated to an amorphous solid under vacuum. This was

dissolved in a minimal amount of boiling chloroform and filtered. The clear concentrated chloroform solution of hemins was then chromatographed as described previously.

Results

The hemins, being highly colored, separate into easily visible bands on the column; they may be collected as they are eluted from the column.

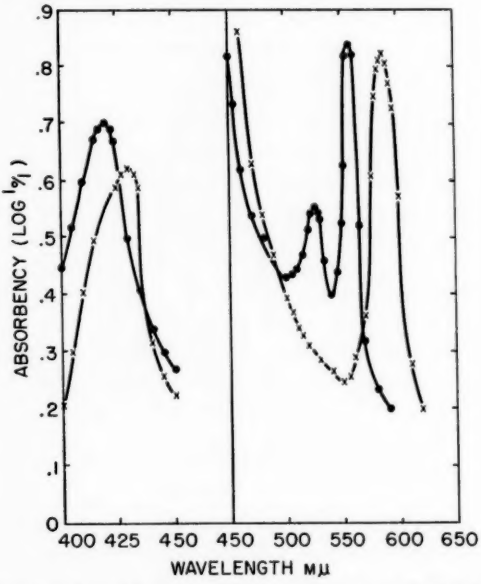


FIG. 2. The reduced pyridine hemochromogen spectra of the hemins prepared from cytochrome oxidase. ●, represents Fraction 1 from the column; ×, represents Fractions 2 and 3. The cuvettes contained the hemin dissolved in 2 ml. of 0.05 N NaOH and 1 ml. of pyridine. The hemochromogen solutions employed for measurements in the visible region were diluted 1:3 for Fraction 1 and 1:5 for Fractions 2 and 3 with alkali and pyridine for measurements in the Soret region.

The eluent may also be collected with an automatic fraction collector and the amount of hemin in successive tubes measured with the Beckman spectrophotometer. All the hemins encountered in this work were found to follow Beer's law of dilution in chloroform at 410 mμ, which was the point of maximal absorption for the *a* type hemins.

Fig. 1 shows a typical chromatograph of the hemins extracted from a cytochrome oxidase preparation. Three hemins, labeled Fractions 1, 2, and 3, are evident. The spectra of the reduced pyridine hemochromogens

of these three hemins appear in Fig. 2. The hemochromogen from Fraction 1 has absorption peaks at 557, 527, and 420 mμ, which are characteristic for pyridine protohemochromogen, and the protohemin undoubtedly arose from the hemoprotein known to be present in the oxidase preparation which absorbs in the region of 560 mμ (16). The hemochromogens of Fractions 2 and 3, however, with absorption peaks at 587 and 430 mμ, are similar to those commonly considered to be derived from hemin *a*.[1] Fractions 2 and 3 are therefore considered to be derived from cytochromes *a* and a_3.

To test the possibility that one of the hemin *a* components gave rise to the other as an artifact of the treatment to which they were subjected, each of the *a* hemins taken from the column was again carried through the entire procedure, including the acid acetone treatment. However, each hemin yielded a single hemin on retreatment, and the product in each case had the same chromatographic and spectral properties as the starting material. Thus these two hemins are not interconvertible during the isolation procedure, which supports the conclusion that they arose from separate hemoproteins.

DISCUSSION

The finding that two hemins of the *a* type may be derived from a cytochrome oxidase preparation leads to the conclusion that they represent the prosthetic groups of the two hemoproteins, cytochromes *a* and a_3. In view of the similar spectral properties of the two *a* type hemins, the different extinction coefficients of cytochromes *a* and a_3 (1, 3–5) and the difference in their abilities to combine with inhibitors must be due more to differences in the linkages between hemin and protein than to the differences in the tetrapyrrole structures. This is supported by the finding that the rates of liberation of the *a* hemins from the hemoproteins of the cytochrome oxidase by acid acetone are very different (17).

The present study supplies no direct evidence as to which hemin *a* is derived from cytochrome a_3. In this connection it is of interest that the areas under the curves in the chromatograph of Fig. 1 indicate approximately equal amounts of the two *a* hemins, and that Chance (5) has estimated that there are essentially equimolar amounts of cytochromes *a* and a_3 in a heart muscle preparation. This comparison seems valid, since it has been shown (16) that the proportions of cytochromes *a* and a_3 are the same in heart muscle particles and in the purified oxidase preparation.

[1] The reduced pyridine hemochromogen of the hemin *a* fraction prepared by Kiese and Kurz (15) possesses minor absorption peaks at 534 and 508 mμ as well as the major ones at 587 and 430 mμ noted by others. These minor peaks are not evident in either of the *a* hemins described in this paper.

The close spectral similarity of the two *a* hemins further indicates that their differences may be related more to the side chains of the hemin nucleus, which are not part of the resonating porphyrin structure, than to essential changes in the porphyrin nucleus itself.

SUMMARY

1. A method for the partition chromatography of hemins is described.

2. Application of the method to the hemins liberated from a purified cytochrome oxidase preparation yielded two hemins of the *a* type. It was concluded that these hemins represent the prosthetic groups of cytochromes *a* and a_3.

3. The relationship of the findings to the difference in properties of cytochromes *a* and a_3 are discussed.

BIBLIOGRAPHY

1. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **127,** 167 (1939).
2. Ball, E. G., Strittmatter, C. F., and Cooper, O., *J. Biol. Chem.*, **193,** 635 (1951).
3. Chance, B., *J. Biol. Chem.*, **202,** 383 (1953).
4. Chance, B., *J. Biol. Chem.*, **202,** 397 (1953).
5. Chance, B., *J. Biol. Chem.*, **202,** 407 (1953).
6. Negelein, E., *Biochem. Z.*, **266,** 412 (1933).
7. Roche, J., and Benevent, M. T., *Bull. Soc. chim. biol.*, **18,** 1650 (1936).
8. Negelein, E., *Biochem. Z.*, **248,** 243 (1932).
9. Negelein, E., *Biochem. Z.*, **250,** 577 (1932).
10. Rawlinson, W. A., and Hale, J. H., *Biochem. J.*, **45,** 247 (1949).
11. Falk, J. E., and Rimington, C., *Biochem. J.*, **51,** 36 (1952).
12. Lemberg, R., and Falk, J. E., *Biochem. J.*, **49,** 674 (1951).
13. Dannenberg, H., and Kiese, M., *Biochem. Z.*, **322,** 395 (1952).
14. Person, P., Wainio, W. W., and Eichel, B., *J. Biol. Chem.*, **202,** 369 (1953).
15. Kiese, M., and Kurz, H., *Biochem. Z.*, **325,** 299 (1954).
16. Smith, L., and Stotz, E., *J. Biol. Chem.*, **209,** 819 (1954).
17. Morrison, M., and Stotz, E., *Federation Proc.*, **12,** 249 (1953).

ON THE NATURE OF OXONIC ACID AND ALLANTOXAIDIN AS OXIDATION PRODUCTS OF URIC ACID AND ALLANTOIN*

By E. S. CANELLAKIS† and PHILIP P. COHEN

(From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin)

(Received for publication, August 12, 1954)

In the course of studies on the chemical structure of purines, Fischer and Ach (1) reported that the chemical oxidation of either 1-methyl- or 7-methyluric acid yields 3-methylallantoin, while under the same conditions the 3-methyl- and 9-methyluric acids yield 1-methylallantoin. On the basis of these results, Behrend (2) assumed that uric acid is oxidized to allantoin by a symmetrical intermediate hydroxyacetylenediureinecarboxylic acid (HDC) (Formula IV). Schuler and Reindel (3) later isolated HDC as a silver salt and showed that the free acid decomposes with the formation of allantoin and CO_2. The occurrence of a symmetrical compound during the chemical and biological oxidation of uric acid has been confirmed in experiments with isotopically labeled compounds (4, 5). The methods used for characterization have been based chiefly on the chemical reduction of allantoin to hydantoin or on its oxidation to potassium oxonate.

The structure of potassium oxonate and of its decarboxylation product allantoxaidin has been a subject of controversy among Biltz and Robl (6), Venable (7), and Moore and Thomas (8). It was finally accepted that these compounds have the structures shown in Fig. 1, as formulated by Biltz.

By the use of uric acids separately labeled with C^{14} in the 2, 5, and 8 positions, evidence has been obtained that these compounds (I, II) cannot have the structures represented. Although conclusive evidence as to the exact structure of these compounds has not been obtained, the accumulated evidence points to a triazine structure.

EXPERIMENTAL

The procedure used for the synthesis of uric acid-2-C^{14} and uric acid-8-C^{14} followed the methods described by Cavalieri *et al.* (9) with few modifications.

* Supported in part by a grant from the Rockefeller Foundation.

† Postdoctoral Fellow of the National Foundation for Infantile Paralysis, 1951–54. Present address, Department of Pharmacology, Yale University, New Haven, Connecticut.

Uric Acid-2-C^{14}—30.5 mg. of urea-C^{14} (total activity = 0.5 mc.) and 169.5 mg. of inert urea were dissolved in 0.6 ml. of absolute methanol containing 323 mg. of potassium methoxide in a 12 × 2 cm. test-tube with a 19/22 standard taper joint. The solution was heated by a nichrome wire coiled around the lower one-fourth of the tube and covered with asbestos tape, and stirred with a 1 cm. Pyrex glass-covered magnetic stirring bar. The reaction mixture was then refluxed and stirred for 4 hours with a solution of 1.3 ml. of absolute methanol containing 730 mg. of acetamidodiethyl malonate, to form acetyluramil. The acetyluramil was hydrolyzed to uramil, which was then condensed with KCNO to pseudouric acid. The pseudouric acid was converted to uric acid and the uric acid-2-C^{14} formed was recrystallized from water. The yield of uric acid-2-C^{14} was 51 per cent with a specific activity of 1.012×10^6 c.p.m. per mg.

Uric Acid-8-C^{14}—350 mg. of uramil were finely ground in a mortar with 40 mg. of urea-C^{14} (total activity = 1 mc.) and 360 mg. of inert urea. The mixture was then fused in the presence of 0.2 ml. of absolute methanol and

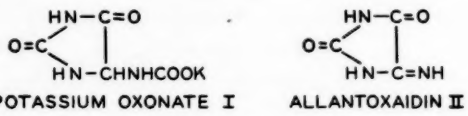


FIG. 1

the pseudouric acid was isolated. The addition of CH_3OH was found to increase the yield of pseudouric acid. This was then converted to uric acid-8-C^{14} (10). Yield of the uric acid-8-C^{14} after recrystallization from water was 46 per cent, calculated on the basis of the uramil used, with a specific activity of 0.965×10^6 c.p.m. per mg.

Uric Acid-5-C^{14}—1.5 gm. of ethyl cyanoacetate containing 0.606 gm. of ethyl cyanoacetate-2-C^{14} (total activity = 0.13 mc.) were condensed with urea in absolute alcohol (11). The 4-aminouracil formed was dissolved in water and reprecipitated with acetic acid. Nitrosation (12, 13) yielded a rose-colored precipitate of 5-nitroso-4-aminouracil, which was then reduced to 4,5-diaminouracil (yield = 1.15 gm.). This was then converted to uric acid by fusion with 1.7 gm. of urea (9). The initial uric acid crystallization yielded 880 mg. of yellow crystals, which were dissolved in 2 liters of boiling water, decolorized with charcoal, and filtered. The filtrate yielded 330 mg. of uric acid-5-C^{14} (over-all yield = 13 per cent) with a specific activity of 22×10^3 c.p.m. per mg.

Both uric acid-2-C^{14} and uric acid-8-C^{14} gave one radioactive peak when eluted from Dowex 1 column under the conditions described in the following publication (14).

The molar extinction coefficients of the uric acid-2-C^{14}, -8-C^{14}, and -5-C^{14} were measured and compared with those reported in the literature (Table I).

Allantoin—Allantoin was prepared from uric acid-2-C^{14}, -8-C^{14}, and -5-C^{14}, according to the procedure of Hartman *et al.* (16) (N found, 35%; theory, 35.4).

Potassium Oxonate—Allantoin, prepared by the oxidation of uric acid-2-C^{14}, -8-C^{14}, and -5-C^{14}, was oxidized to potassium oxonate according to the method of Biltz and Giesler (17) and recrystallized from water (N found, 22.4%; theory, 22.3). Potassium oxonate was also prepared from allantoic acid, following the identical procedure used for its synthesis from allantoin (N found, 22.2%; theory, 22.3).

TABLE I

Molar Extinction Coefficients of C^{14}-Uric Acids

Sample	E_{292}	E_{260}	E_{235}	Ratio, E_{292}/E_{235}
Uric acid-2-C^{14}	12,520	2910	10,230	1.223
" acid-8-C^{14}	12,310	2810	9,900	1.245
" acid-5-C^{14}	11,500	2750	9,265	1.238
Recrystallized commercial	12,020	2578	8,370	1.435
Cavalieri *et al.* (9)	12,700		10,100	1.256
Stimson-Reuter (15)	12,200		9,800	1.235

Hydantoin—Hydantoin was prepared by reduction of allantoin with HI according to the method of Baeyer (18), as modified by Cavalieri *et al.* (9) (N found, 27.8%; theory, 27.6).

RESULTS AND DISCUSSION

As can be seen from Table II, the molar specific activities of the allantoin and potassium oxonate, when prepared from uric acid-2-C^{14}, -8-C^{14}, or -5-C^{14}, are the same as those of the labeled uric acids from which they are derived. The CO_2 arising from the decarboxylation of potassium oxonate derived from uric acid-2-C^{14} or -8-C^{14} is inactive. In contrast, the CO_2 which arises by decarboxylation of the potassium oxonate derived from uric acid-5-C^{14} has the same molar specific activity as its corresponding uric acid, allantoin, and potassium oxonate.

The hydantoin arising from the reductive cleavage of allantoin derived from uric acid-2-C^{14} or -8-C^{14} contains one-half the radioactivity of allantoin, whether the allantoin was prepared from uric acid-2-C^{14} or -8-C^{14}.

It is generally accepted from the work of Biltz (19) and Schuler and Reindel (3) that, when uric acid (III) is oxidized by $KMnO_4$ in alkaline

solution, a symmetrical intermediate hydroxyacetylenediureinecarboxylic acid (HDC) is formed (IV). This symmetrical intermediate (HDC) is stable in basic solution for a short period of time. Upon acidification, it decarboxylates to give allantoin (VI), according to the series of reactions shown in Fig. 2.

TABLE II

Specific Activity of Derivatives and Degradation Products Derived from Uric Acid

	Uric acid-2-C^{14}	Uric acid-8-C^{14}	Uric acid-5-C^{14}
	c.p.m. per μmole	*c.p.m. per μmole*	*c.p.m. per μmole*
Uric acid	172.0	162.1	92.3
Allantoin	170.1	164.0	94.0
Potassium oxonate	172.9	160.4	90.9
CO_2 (isolated as $BaCO_3$)	0	0	88.0
Hydantoin	85.2	79.3	

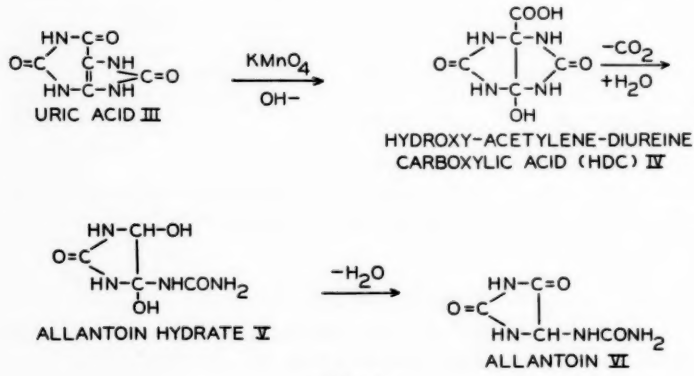


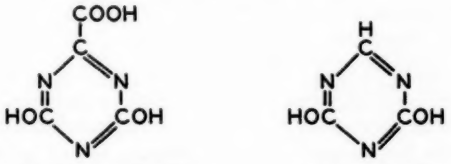
FIG. 2

Biltz (19) postulated a concurrent decarboxylation and hydration of HDC at carbon 5 to yield (V). Upon subsequent dehydration of (V), the carbonyl carbon of allantoin (VI) is formulated as being derived from carbon 5 of uric acid (III). If uric acid labeled with C^{14} in position 2 or 8 is oxidized, owing to the symmetry of the intervening HDC, the C^{14} would be expected to be equally distributed between the imidazole ring and the ureide group of allantoin. Accordingly, if potassium oxonate had the formula (I) attributed to it by Biltz, it should, upon decarboxylation to allantoxaidin, yield radioactive CO_2 containing one-half the radioactivity of the allantoin or oxonate from which it was derived. The data of Table

II clearly reveal that CO_2 does not arise from carbon 2 or 8, as would have been expected in accordance with Biltz's formulation of potassium oxonate, but does arise from carbon 5.

In alkaline solution, allantoin is known to form allantoic acid (20). Because allantoic acid also forms potassium oxonate under the same conditions as those used for the oxidation of allantoin, it is not improbable that allantoic acid is an intermediate in this reaction. Since allantoic acid is a symmetrical compound, equilibration of the 2-C^{14} and 8-C^{14} label can occur in this alkaline medium. Because of these considerations, this type of degradation may not be suitable for the study of N^{15}, 2-C^{14}, or 8-C^{14} distribution in uric acid, and interpretations based upon its use (4) must now be held in question.

Oxidation of allantoic acid could lead to the formation of 2,4-dihydroxytriazine-6-carboxylic acid (VII) in which the carboxyl group would be C-5



2,4 DIHYDROXYTRIAZINE-6-CARBOXYLIC ACID (VII)

2,4 DIHYDROXY-TRIAZINE (VIII)

FIG. 3

of the original uric acid.[1] Upon decarboxylation, 2,4-dihydroxytriazine (VIII) would be formed (Fig. 3). In favor of this formulation is (1) the ease of oxidation of oxonic acid to cyanuric acid by H_2O_2, as shown by Biltz and Robl (6), and (2) the decarboxylation of potassium oxonate with the loss of carbon 5 derived from uric acid.

The reductive cleavage of allantoin to hydantoin with HI shows a symmetrical distribution of radioactivity between the ureide group and the imidazole ring. It should be recognized that allantoin may itself be an inherently symmetrical compound through tautomerization. The answer to the question as to whether the HI reaction can be used for degradative studies on isotopically labeled allantoin and whether allantoin tautomerizes via a symmetrical intermediate must await synthesis of allantoin by procedures other than the oxidation of uric acid.

[1] The fact that carbons 2 and 8 of uric acid do not yield radioactive CO_2 when K oxonate is decarboxylated to allantoxaidin has been independently established by Dr. Hans Brandenberger, University of Berne, Switzerland, who has also shown that carbons 4 and 6 do not yield radioactive CO_2 (personal communication). Dr. Brandenberger's excellent and detailed study of this problem has been published since this manuscript was submitted (21).

SUMMARY

The formulas proposed by Biltz and coworkers for potassium oxonate and allantoxaidin appear to be incorrect. Evidence from degradation studies of isotopically labeled compounds suggests a triazine ring structure for these compounds.

BIBLIOGRAPHY

1. Fischer, E., and Ach, F., *Ber. chem. Ges.*, **32,** 2721 (1899).
2. Behrend, R., *Ann. Chem.*, **333,** 153 (1904).
3. Schuler, W., and Reindel, W., *Z. physiol. Chem.*, **215,** 258 (1933).
4. Brown, G. B., Roll, P. M., and Cavalieri, L. F., *J. Biol. Chem.*, **171,** 835 (1947).
5. Cavalieri, L. F., and Brown, G. B., *J. Am. Chem. Soc.*, **70,** 1242 (1948).
6. Biltz, H., and Robl, R., *Ber. chem. Ges.*, **53,** 1967 (1920).
7. Venable, C. S., *J. Am. Chem. Soc.*, **40,** 1099 (1918).
8. Moore, F. J., and Thomas, R. M., *J. Am. Chem. Soc.*, **40,** 1120 (1918).
9. Cavalieri, L. F., Blair, V. E., and Brown, G. B., *J. Am. Chem. Soc.*, **70,** 1240 (1948).
10. Fischer, E., *Ber. chem. Ges.*, **30,** 559 (1897).
11. Conrad, M., *Ann. Chem.*, **340,** 310 (1905).
12. Traube, W., *Ber. chem. Ges.*, **33,** 1382 (1900).
13. Bogert, M. T., and Davidson, D., *J. Am. Chem. Soc.*, **55,** 1667 (1933).
14. Canellakis, E. S., and Cohen, P. P., *J. Biol. Chem.*, **213,** 385 (1955).
15. Stimson, M. M., and Reuter, M. A., *J. Am. Chem. Soc.*, **65,** 153 (1943).
16. Hartman, W. W., Moffett, E. W., and Dickey, J. B., *Org. Syntheses*, coll. **2,** 21 (1943).
17. Biltz, H., and Giesler, E., *Ber. chem. Ges.*, **46,** 3410 (1913).
18. Baeyer, A., *Ann. Chem.*, **130,** 158 (1864).
19. Biltz, H., *J. prakt. Chem.*, **145,** 65 (1936).
20. Behrend, R., and Schultz, R., *Ann. Chem.*, **365,** 36 (1909).
21. Brandenberger, H., *Biochim. et biophys. acta*, **15,** 18 (1954).

THE END-PRODUCTS AND INTERMEDIATES OF URIC ACID OXIDATION BY URICASE*

By E. S. CANELLAKIS† and PHILIP P. COHEN

(From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin)

(Received for publication, August 12, 1954)

Since the early work on uricase carried out by Schittenhelm (1), Wiechowski (2), and Battelli and Stern (3), and the elucidation of the structure of uric acid by the work of Behrend and Roosen (4), Fischer (5), and Biltz (6), the chemical nature of the intermediates and end-products of uricase action has been uncertain. The first indications of the structure of a possible intermediate were offered by Schuler and Reindel (7), who were able to isolate, from a uricase system, a silver salt with properties similar to those of the silver salt of hydroxyacetylenediureinecarboxylic acid (HDC) (Formulas V, VI, Fig. 1) which they (8) had previously isolated during the chemical oxidation of uric acid. The existence and structure of such a symmetrical intermediate had been postulated by Behrend (9), who had not been able to effect its isolation. Klemperer (10) concluded from his studies on uricase action that the primary product of uric acid oxidation is an unstable compound which decomposes non-enzymatically to form allantoin, uroxanic acid, and HDC. The relative amounts of these three compounds were found to depend on the pH and the buffer used. Praetorius (11) obtained spectrophotometric evidence for the formation during the action of uricase on uric acid of an unstable intermediate (maximal absorption, 300 to 330 mμ) which, he concluded, was a precursor of HDC. Bentley and Neuberger (12), using O^{18}, H_2O^{18}, and 6-C^{14}-labeled uric acid, concluded that uricase catalyzes the transfer of 2 electrons from the urate ion, and proposed a mechanism for the enzymatic oxidation of uric acid leading to HDC.

Although Wiechowski (2) had considered uricase to catalyze the oxidation of uric acid quantitatively to allantoin, Schuler (13) could account for only 18 per cent of the expected CO_2 production at pH 8.9 in borate buffer, and Davidson (14), using a purified uricase preparation in borate buffer at pH 9, could not account for more than 30 per cent of the added uric acid as allantoin. Klemperer (10) also found that only a fraction of

* Supported in part by a grant from the Rockefeller Foundation.

† Postdoctoral Fellow of the National Foundation for Infantile Paralysis, 1951–54. Present address, Department of Pharmacology, Yale University, New Haven, Connecticut.

the theoretical CO_2 was evolved during the enzymatic oxidation of uric acid.

In this paper, evidence will be presented which indicates that in borate buffer at least three unstable intermediate compounds are formed. We have been able to elucidate the formula of one of these intermediates and have shown that urea and alloxanic acid are formed in addition to allantoin as end-products. In phosphate buffer at pH 7.2 and 8.4, the products of uricase action can be accounted for mainly as allantoin.

EXPERIMENTAL

Synthesis of Labeled Uric Acid—The synthesis and the properties of the 2-C^{14}- and the 8-C^{14}-labeled uric acids used in these experiments have been described in the previous communication (15).

Uricase—Uricase was prepared according to Altman, Smull, and Barron (16) through Step II of their method. The enzyme was then precipitated

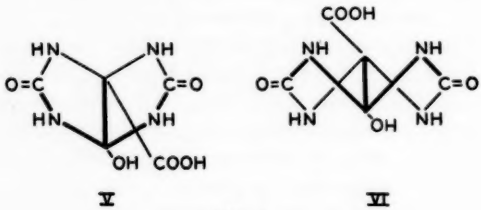


FIG. 1

with ammonium sulfate and the precipitate dissolved in 0.1 M phosphate buffer, pH 7.2 (17). The enzyme preparation had an activity based on oxygen uptake of 115 μl. of O_2 per 0.5 ml. of enzyme solution in 30 minutes when 1.7 mg. of uric acid were incubated in the presence of 0.1 M phosphate buffer, pH 7.2, in a final volume of 2.5 ml. The soluble preparation was stored at $-20°$ and used as needed. It was found to contain catalase activity but no measurable peroxidase activity.[1]

Incubation Conditions—0.5 ml. of 0.4 M phosphate buffer, pH 7.1, was added to 1.7 mg. (10.1 μmoles) of C^{14}-labeled uric acid. The suspension was allowed to stand for a few minutes. 1.5 ml. of water were then added and the uric acid brought into solution with stirring. 0.5 ml. of the uricase solution was then added, the flask was flushed with oxygen, and the incubation carried out at 38° with shaking for 30 minutes. In experiments in which a higher pH was required, a calculated amount of NaOH solution was added in place of water.

In the borate experiments 1.7 mg. of C^{14}-labeled uric acid were dissolved in 1 ml. of 0.26 M borate buffer, pH 7.2, with the addition of 1.0 ml. of

[1] We wish to thank Mr. E. A. Steberl for the assays of peroxidase activity.

water as described above. The incubation was carried out as previously indicated in the presence of 0.5 ml. of uricase solution. The final borate concentration was 0.104 M, and the final phosphate concentration (due to the phosphate buffer in the uricase solution) was 0.02 M. In the borate experiments carried out at pH 9.0, the pH was adjusted by the addition of NaOH solution. The pH was checked at the beginning and end of incubation.

Column Chromatography—10 or 20 cm. columns of Dowex 1 in the formate form were used. The elution was carried out according to Hurlbert *et al.* (18) with 1 M ammonium formate, pH 5 to 6 in the reservoir, and beginning with a 200 ml. volume of water in the mixing flask. After incubation of the C^{14}-labeled uric acid with uricase under the specified conditions of the experiment, the incubation mixture was added to the column and eluted with increasing concentrations of ammonium formate. The total eluted radioactivity in various experiments was found to account for 90 to 105 per cent of that added to the column. The fractions corresponding to radioactive peaks were then isolated and further identified as follows.

Urea—Urea was identified chromatographically with paper chromatography and by the urease reaction. The latter reaction was also used for a quantitative determination of the radioactivity in the urea by collecting and isolating the evolved CO_2 as $BaCO_3$.

Allantoin—Allantoin was determined by the carrier technique and recrystallization to constant specific activity. It was then oxidized to K oxonate (19) and the specific activity redetermined. Identification was further confirmed by paper chromatography.

Alloxanic Acid—Alloxanic acid was identified by (1) recrystallization to constant specific activity with carrier alloxanic acid, (2) oxidation to parabanic acid (20) and isolation of ammonium oxalurate (21), (3) conversion of ammonium oxalurate to oxaluric acid (21), (4) paper chromatography, and (5) the coincidence of the radioactivity peak and the ultraviolet absorption at E_{250} when a mixture of isolated radioactive alloxanic acid was eluted with carrier synthetic alloxanic acid through a 20 cm. Dowex 1 column under the previously specified conditions.

Incubation of Uric Acid in Phosphate Buffer—Incubations were carried out at pH 7.2 and 8.5. As can be seen from Table I, in both cases allantoin was the major end-product accounting for 93 to 95 per cent of the total eluted activity. Urea was found to account for between 1 and 2 per cent of the total eluted activity. About 2 per cent of the total added activity was found to be firmly attached to the column, but could be eluted with 6 M formic acid.

On column chromatograms the allantoin peak was accompanied by a small but discrete peak (25 to 30 ml. of effluent volume), the component of

which behaved like allantoin on paper chromatograms and by carrier technique isolation. Upon rechromatography of this fraction on a Dowex 1 column, it was found to coincide with allantoin. This compound has not been further identified, but for the purpose of this discussion has been termed "allantoin precursor."

TABLE I

*Products of Uricase Action in Phosphate Buffer**

pH	Substrate	Urea	Allantoin	Allantoin precursor
7.2	Uric acid-2-C^{14}	1	95	4–5
7.2	" acid-8-C^{14}	1–2	94	1–2
8.5	" acid-2-C^{14}	1–2	93	4–5

* Values expressed as per cent of the total eluted radioactivity.

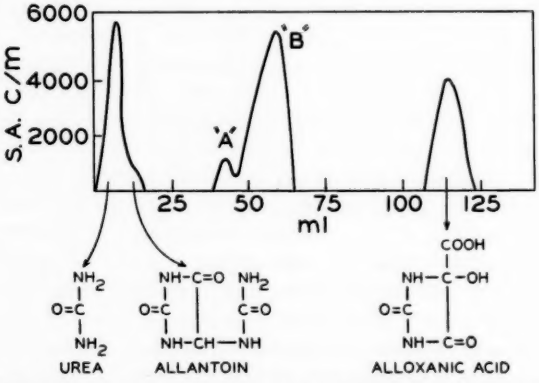


FIG. 2. Column chromatograph diagram of end-products resulting from incubation of 2-C^{14}-labeled uric acid with uricase for 30 minutes in borate buffer, pH 7.2. For an explanation, see the text.

Incubation of Uric Acid in Borate Buffer, pH 7.2—There is a marked qualitative and quantitative difference in the end-products when uricase is incubated with uric acid in the presence of borate buffer, as compared with those obtained in phosphate buffer.

Fig. 2 represents diagrammatically the results of an experiment in which 2-C^{14}-labeled uric acid was incubated with uricase for 30 minutes in borate buffer at pH 7.2. As can be seen, the amount of urea formed is greatly enhanced, while the amount of allantoin formed is diminished. In addition, alloxanic acid and two other components are present, as revealed by Peaks A and B.

Alloxanic acid has been identified as previously mentioned. Recrystallization experiments of alloxanic acid and its derivatives gave specific activities of 220, 217, and 206 c.p.m. per μmole respectively for potassium alloxanate, ammonium oxalurate, and oxaluric acid. Table II shows that the R_F values of radioactive alloxanic acid, upon paper chromatography, coincide with those of synthetic alloxanic acid in three different solvent systems.

TABLE II

Identification of Alloxanic Acid by Paper Chromatography

Solvent (volume by volume)	R_F of isolated radioactive alloxanic acid	R_F of synthetic alloxanic acid
95% ethanol-80% formic acid (12.5:2)	0.25	0.25
n-Butanol-ethylene glycol-80% formic acid-water (3:1:1:1)	0.54	0.54
n-Butanol-acetic acid-water (4:1:1)	0.15	0.15

TABLE III

*Asymmetric Decomposition of UIDC (Peak B) upon Rechromatography through Dowex 1 Column**

UIDC obtained from	Recovered as†	
	Urea	Alloxanic acid
Uric acid-2-C^{14}	0	100
" acid-8-C^{14}	100	0

* Upon isolation of UIDC from the incubation mixture, it was taken up in phosphate buffer, pH 7.2, and rechromatographed.

† Values expressed as per cent of total eluted radioactivity.

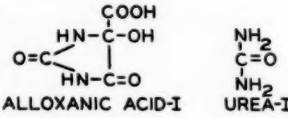
The radioactivity represented by Peak A is due to an unstable compound which decomposes upon rechromatography. Because of the small amounts available, we have not been able to identify this compound further. The radioactivity represented by Peak B is due to an unstable compound which decomposes asymmetrically to urea and alloxanic acid.

Subsequent to the incubation of uric acid-2-C^{14} with uricase, the radioactive compound represented by Peak B decomposes into radioactive alloxanic acid and non-radioactive urea. In contrast, after the incubation of uric acid-8-C^{14} with uricase, the radioactive compound represented by Peak B decomposes into radioactive urea (Table III).

As shown in Fig. 3, the compound represented by Peak B may have the

formula represented by the summation of these two compounds (Formula III). It seems probable that this compound, 5-ureido-2-imidazolidone-4,5-diol-4-carboxylic acid (UIDC), due to its adjacent hydroxyl groups has been stabilized as the borate complex, as indicated in Formula IV.

The stabilization of UIDC by borate has been shown by collecting UIDC during the chromatographic elution (see Table IV) in tubes which contain



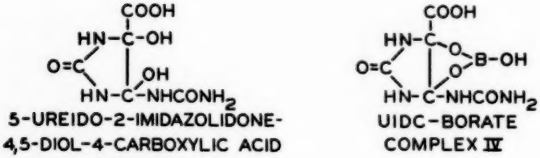


FIG. 3

TABLE IV

Stabilization of UIDC (Peak B) from Uric Acid-8-C^{14} by Borate Buffer

UIDC, collected in presence of	Per cent accounted for,* upon rechromatography, as	
	UIDC	Urea
Borate buffer, pH 7.2	91	9
Phosphate buffer, pH 7.2	0	100

*Values expressed as the per cent of total eluted radioactivity.

borate buffer (final concentration 0.1 M, pH 7.2). When this fraction is then immediately put through another Dowex 1 column, UIDC could be removed unchanged. In the absence of borate buffer in the collecting tubes and in the presence of phosphate (final concentration 0.1 M, pH 7.2), the radioactivity was accounted for as urea or alloxanic acid.

The quantitative changes in the end-products with time can be seen in Table V. When uric acid-2-C^{14} is incubated with uricase, the decrease in radioactivity of UIDC can be mainly accounted for by the radioactive alloxanic acid formed. When uric acid-8-C^{14} is incubated with uricase, the decrease in radioactivity of UIDC can be accounted for by the radio-

active urea formed. This is in accord with the non-enzymatic decomposition of UIDC shown above.

Incubation of Uric Acid in Borate Buffer, pH 9.0—Qualitatively the picture of uric acid oxidation by uricase in borate buffer at pH 9.0 (Table VI) is similar to that observed in borate buffer at pH 7.2. The quantitative aspects are quite different since, as can be seen, most of the allantoin appears as the "allantoin precursor" mentioned earlier. The allantoin precursor

TABLE V

*Products of Uricase Action on 2-C^{14}- and 8-C^{14}-Labeled Uric Acids in Borate Buffer, pH 7.2**

Incubation time	C^{14} products formed from uric acid labeled in									
	C-2	C-8	C-2	C-8	C-2	C-8	C-2	C-8	C-2	C-8
	Urea		Allantoin		Peak A		Peak B		Alloxanic acid	
min.										
30	20	25	12	12	4	6	42	40	24	15
60	22	45	16	13	4	4	24	20	35	17
90	22		14		3		18		45	

* Values expressed as per cent of the total eluted radioactivity.

TABLE VI

*Products of Uricase Action on Labeled Uric Acid in Borate Buffer, pH 9.0**

Substrate	Urea	Allantoin	Allantoin precursor	Peak A	Peak B	Alloxanic acid
Uric acid-2-C^{14}.....	30	3	29	7	4	28
" acid-8-C^{14}.....	30	3	29	6	4	29

* Individual values expressed as per cent of the total eluted radioactivity.

seems to be formed at the expense of UIDC. It should be noted that alloxanic acid is formed in approximately the same amounts, whether uric acid-2-C^{14} or -8-C^{14} is used.

DISCUSSION

HDC has been accepted since Schuler and Reindel's (7, 8) work to be an intermediate during both the chemical and the enzymatic oxidation of uric acid. The evidence reported in this paper for the structure of UIDC as a monohydrated form of HDC in turn provides additional evidence for the occurrence of HDC as an intermediate during the enzymatic oxidation of uric acid.

HDC could theoretically exist in two forms corresponding to cis and trans forms of the COOH and OH groups (Fig. 1, V and VI).

From the evidence of Barrett and Linstead (22) on 0:3:3 β-bicyclooctanones, which have five-membered ring structures similar to HDC, the occurrence of the trans form would be very improbable because of the exceptionally large difference in heats of combustion (6.8 kilocalories) which places the trans form at a much higher energy level. Further discussion will therefore be limited to the assumption that we are dealing only with the cis form of HDC.

The existence of a compound like UIDC makes it apparent that the further cleavage of HDC is hydrolytic. The asymmetry of the breakdown products of UIDC indicates that this hydrolytic cleavage occurs preferentially between C-5 and N-7. Because of the impurity of the uricase preparation, it is difficult to exclude the existence of a specific hydrolytic enzyme. The existence of such an enzyme could explain the asymmetric hydrolytic cleavage of HDC as an Ogston type compound based on the three point attachment theory.

The asymmetric distribution of radioactivity of UIDC may also find an explanation based on known chemical reactions of uric acid. Fischer and Ach (23) have shown that either 1- or 7-monomethyluric acid is oxidized by $KMnO_4$ to 3-methylallantoin,[2] while 7- or 9-monomethyluric acid under similar conditions yields 1-methylallantoin. Based on this work, Behrend (9) postulated the intermediate formation of HDC. The common characteristic of these reactions is that the existence of a methyl group on either the pyrimidine or the imidazole moiety of uric acid apparently stabilizes the ring so that the allantoin formed is in all cases methylated in the ring. The cleavage of the methylated HDC intermediate must have, therefore, occurred mainly in the non-methylated ring.

If it is assumed that the enzymatic oxidation occurs via the pyrimidine moiety of uric acid because of the closer attachment of this ring to the uricase surface or prosthetic group, further hydration of the intermediate would be expected to occur, in conformance with Fischer and Ach's results, preferentially between C-5 and N-7.

Hydration of the cis form of HDC between C-5 and N-7 would lead to the formation of UIDC with 2 asymmetric carbons, carbons 4 and 5. As a consequence, four optical isomers corresponding to the spatial arrangement of the substituents of carbons 4 and 5 could be formed. Two of these would have *cis*-hydroxyl groups while the hydroxyl groups of the other two would be in the trans position. The cis form can then be stabilized by borate ions to yield asymmetrically labeled UIDC. The trans forms, which cannot be stabilized by borate ion, would decompose to yield labeled alloxanic acid

[2] The numbering of allantoin is that corresponding to a 5-ureidohydantoin.

and unlabeled urea from uric acid-2-C^{14}. Conversely the amount of labeled urea formed from uric acid-2-C^{14} would be a measure of the extent to which the hydration of HDC occurred between C-5 and N-1.

Further evidence for this asymmetric breakdown of uric acid has been sought by degrading the allantoin isolated from systems containing uricase in phosphate and borate buffers. The hydriodic acid degradation to hydantoin (Table VII) resulted in equivalent labeling of the imidazole ring and the ureide chain of allantoin. As discussed in the previous paper (15), this may be a reflection of the inherent symmetry of the allantoin molecule and not of the symmetrical breakdown of HDC.

The stabilization of UIDC by borate buffer can be seen from the data of Table IV. When the UIDC fraction is eluted into tubes containing borate buffer at pH 7.2, it can be recovered upon rechromatography in the same

TABLE VII

Distribution of C^{14}-Radioactivity after Reduction of Allantoin to Hydantoin

Source of allantoin*	Allantoin	Hydantoin
	c.p.m. per μmole	*c.p.m. per μmole*
Phosphate buffer, pH 7.2	163	79
Borate buffer, pH 7.2	23.5	12.8

* Allantoin obtained after oxidation of uric acid-2-C^{14} by uricase under the specified conditions.

form. In contrast, when it is collected in the absence of borate buffer, only its degradation products are found.

At pH 9.0 in borate buffer, there is an increase in the "allantoin precursor" formed. Since the allantoin precursor has the properties of allantoin, it can be assumed that the chemical isolation of allantoin from such a system would include allantoin plus the allantoin precursor. In this system the total allantoin can therefore account for about 30 per cent of the total uric acid decomposition. This is in agreement with the findings of other investigators (10, 13, 14) that only a fraction of the uric acid decomposed can be accounted for as allantoin when uric acid is incubated with uricase in borate buffer at pH values of approximately 9.0.

Under these conditions the amount of alloxanic acid formed is equivalent to that of urea, while UIDC accounts for only 4 per cent of the total activity. It seems that borate buffer at pH 9.0 favors the formation of the allantoin precursor to that of UIDC. The structure of the allantoin precursor has not been determined in the present study. Some insight as to its structure might be gained by the use of uric acid-6-C^{14}, since it would reveal whether the allantoin precursor still has C-6 and whether it could be HDC.

XUM

The data presented on the action of uricase on uric acid in phosphate buffer differ from those of Klemperer in that no relationship between pH and the amount of allantoin formed has been observed, nor any indication of the formation of appreciable amounts of uroxanic acid. The results of the present study indicate that in phosphate buffer uricase catalyzes the oxidation of uric acid mainly to allantoin. The end-products remain quantitatively similar at pH 7.2 and 8.5.

These experiments have provided evidence for the existence of at least three different unstable intermediates, the allantoin precursor, a compound corresponding to Peak A, and UIDC, which are formed during the oxidation of uric acid by uricase. The chemical structure of the first two remains to be determined, as well as the interrelationship of these three compounds.

SUMMARY

1. The action of uricase has been studied in the presence of phosphate and borate buffers at various pH values, with the use of 2-C^{14}- and 8-C^{14}-labeled uric acids.

2. In the presence of phosphate buffer and at pH 7.2 or 8.5, uric acid is mainly oxidized to allantoin.

3. In the presence of borate buffer at pH 7.2, the main products are urea and alloxanic acid. An unstable intermediate, apparently 5-ureido-2-imidazolidone-4,5-diol-4-carboxylic acid (UIDC), is also formed and decomposes to urea and alloxanic acid.

4. The end-products of uric acid degradation in borate buffer, pH 9.0, are urea, allantoin, and alloxanic acid. The formation of "allantoin precursor," an unidentified unstable intermediate, is favored in this system over that of UIDC.

5. The chemistry and possible modes of formation of these compounds are discussed.

BIBLIOGRAPHY

1. Schittenhelm, Z., *Z. physiol. Chem.*, **46,** 121 (1905).
2. Wiechowski, W., *Beitr. chem. Physiol. u. Path.*, **9,** 295 (1907).
3. Battelli, F., and Stern, L., *Biochem. Z.*, **19,** 219 (1909).
4. Behrend, R., and Roosen, O., *Ann. Chem.*, **251,** 235 (1889).
5. Fischer, E., Untersuchungen in der Puringruppe (1882–1906), Berlin (1907).
6. Biltz, H., *J. prakt. Chem.*, **145,** 65 (1936).
7. Schuler, W., and Reindel, W., *Z. physiol. Chem.*, **215,** 258 (1933).
8. Schuler, W., and Reindel, W., *Z. physiol. Chem.*, **208,** 248 (1932).
9. Behrend, R., *Ann. Chem.*, **333,** 153 (1904).
10. Klemperer, F. W., *J. Biol. Chem.*, **160,** 111 (1945).
11. Praetorius, E., *Biochim. et biophys. acta*, **2,** 602 (1948).
12. Bentley, R., and Neuberger, A., *Biochem. J.*, **52,** 694 (1952).

13. Schuler, W., *Z. physiol. Chem.*, **208,** 237 (1932).
14. Davidson, J. N., *Biochem. J.*, **36,** 252 (1942).
15. Canellakis, E. S., and Cohen, P. P., *J. Biol. Chem.*, **213,** 379 (1955).
16. Altman, K. I., Smull, K., and Barron, E. S. G., *Arch. Biochem. and Biophys.*, **21,** 158 (1949).
17. Tuttle, A. L., Thesis, University of Wisconsin (1953).
18. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209,** 23 (1954).
19. Biltz, H., and Giesler, E., *Ber. chem. Ges.*, **46,** 3410 (1913).
20. Biltz, H., Heyn, M., and Bergius, M., *Ann. Chem.*, **413,** 70 (1917).
21. Wöhler, F., and Liebig, J., *Ann. Chem.*, **26,** 241 (1838).
22. Barrett, J. W., and Linstead, R. P., *J. Chem. Soc.*, 436 (1935).
23. Fischer, E., and Ach, F., *Ber. chem. Ges.*, **32,** 2721 (1899).

A COMPARATIVE STUDY OF THE END-PRODUCTS OF URIC ACID OXIDATION BY PEROXIDASES*

By E. S. CANELLAKIS,† ALICE L. TUTTLE,‡ AND PHILIP P. COHEN

(From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin)

(Received for publication, August 25, 1954)

Uric acid has long been considered to be a metabolically inert end-product of purine metabolism in the human (1, 2). This view has been challenged by Folin, Berglund, and Derick (3), who, in an extensive review of the subject, reported that only about 50 per cent of intravenously administered uric acid in man could be recovered. More recently, with the use of N^{15}-labeled uric acid, Benedict, Forsham, and Stetten (4) calculated that only 70 to 80 per cent of the uric acid formed each day by normal humans could be isolated in the urine. In more recent experiments, Wyngaarden and Stetten (5) have shown that, after administering 1000 mg. of uric acid-1,3-N^{15} to a normal human, 17 per cent of the administered N^{15} could be recovered as urinary urea and 1 per cent as urinary ammonia, thus establishing uricolysis in the human. These may be considered to be low values, since they do not take into consideration the initially high elimination of the administered uric acid owing to the large dose administered. The questions are therefore raised as to what enzyme systems are involved, the mechanism of uric acid oxidation, and the end-products of this degradation.

In the present investigation it will be shown that uric acid can be oxidized in the presence of the following enzyme systems: lactoperoxidase, verdoperoxidase, horseradish peroxidase, and catalase. Some aspects of the mechanism of these oxidations have been studied and the end-products identified.

EXPERIMENTAL

2-C^{14}- and 8-C^{14}-labeled uric acids with specific activities of 1.02×10^6 c.p.m. per mg. and 0.9×10^6 c.p.m. per mg., respectively, were prepared as described in a previous communication (6). Lactoperoxidase was prepared by the method of Polis and Shmukler (7) up to the stage prior to

* Supported in part by a grant from the Rockefeller Foundation.

† Postdoctoral Fellow of the National Foundation for Infantile Paralysis, 1951–54. Present address, Department of Pharmacology, Yale University, New Haven, Connecticut.

‡ Present address, Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California.

XUM

crystallization.[1] The protein concentration was 7.5 mg. per ml. of activity, and 1056.6 units per ml. were measured according to the method of Schultz *et al.* (8). Because of the method of preparation, borate is present as a contaminant in this lactoperoxidase preparation. Verdoperoxidase prepared from rat chloroma tissue (8) was obtained through the courtesy of Dr. J. Schultz, Temple University, Philadelphia. The protein concentration of this preparation was 3.7 mg. per ml.; activity, 370 units per ml. (8). Crystalline catalase was prepared by a slight modification of the method of Tauber and Petit (9). It had an activity of 14 catalase units per ml. (10). Horseradish peroxidase was obtained through the courtesy of Dr. R. H. Kenten, Rothamsted Experimental Station, Harpenden, Herts, England. It was prepared by the method of Kenten and Mann (11), and had an activity of purpurogallin number = 1040. Notatin (glucose oxidase) (batch No. N/14) was obtained from Boots Pure Drug Company, Ltd., Nottingham, England. Ethyl hydrogen peroxide (EtOOH) was prepared according to the method of Baeyer and Villiger (12). The initial distillate was made basic with KOH and redistilled until one-third of the volume was left. The residue was then acidified, distilled, and collected in three fractions. It was used as a 10 to 15 per cent aqueous solution. Oxalyl diurea was prepared from allantoin according to the method of Biltz and Topp (13) (N found, 30.0%; theory, 30.4). Carbonyl diurea was prepared from uric acid according to the method of Ohta (14) (N found, 38.3%; theory, 38.6). Cyanuric acid was prepared by refluxing cyanuryl chloride for 5 hours with a large excess (1:30) of glacial acetic acid. It was allowed to stand at room temperature overnight, filtered, and recrystallized from water (N found, 32.5%; theory, 32.6). Parabanic acid was prepared by Asche's (15) modification of Menschutkin's (16) procedure (N found, 24.1%; theory, 24.5). Ammonium oxalurate was prepared from parabanic acid according to the method of Wöhler and Liebig (17) (N found, 28.5%; theory, 28.2). Oxaluric acid was obtained from ammonium oxalurate by acidification (17) (N found, 21.4%; theory, 21.2).

The chromatographic techniques previously described (18) as well as crystallization to constant specific activity were used for the identification of the uric acid breakdown products.

Incubation Conditions; Catalase—0.4 ml. of catalase and 0.4 ml. of EtOOH were added to a 2.5 ml. solution of 1.5 mg. of labeled uric acid in 0.1 M phosphate buffer, pH 7.2; final pH, 6.7 to 6.8; incubation at 38° for 30 minutes.

Lactoperoxidase and Verdoperoxidase—110 units of lactoperoxidase or verdoperoxidase were incubated with 5 mg. of notatin, 10 mg. of glucose,

[1] The purified lactoperoxidase was prepared by Mr. E. A. Steberl of this Department.

and 1 mg. of labeled uric acid in 3.0 mg. of 0.1 M phosphate buffer, pH 7.3, for 1 hour at 38°; final pH, 7.1.

Horseradish Peroxidase—1 mg. of horseradish peroxidase was incubated with 1 mg. of uric acid-2-C^{14} under the same conditions as described for lactoperoxidase and verdoperoxidase.

Results

Catalase Plus Ethyl Hydrogen Peroxide—No appreciable decomposition of uric acid could be demonstrated spectrophotometrically in the presence of catalase and a hydrogen peroxide-generating system composed of notatin and glucose at pH 7.2. Keilin and Hartree (19) reported that ethyl hydrogen peroxide could be used in the presence of catalase to oxidize ethanol to acetaldehyde. Preliminary experiments indicated no effect of catalase or EtOOH alone on uric acid. Spectrophotometric data revealed that incubation of uric acid with catalase and EtOOH in 0.1 M phosphate buffer, pH 7.2, led to a rapid disappearance of the 293 mμ uric acid peak and to the concurrent appearance of an absorption peak at 325 mμ (20).

Incubation of uric acids-2-C^{14} and -8-C^{14} with the catalase-EtOOH system led to a large number of oxidation products (Table I) which were identified. It will be noticed that a common characteristic of these compounds is the absence of C-6 of uric acid, indicating that their formation took place subsequent to the loss of this carbon.

Radioactive parabanic acid and its open ring form, oxaluric acid, could be isolated only from the catalase-EtOOH system in which uric acid-8-C^{14} was oxidized.

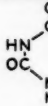
Lactoperoxidase—Lactoperoxidase (which contains borate as a contaminant), when incubated in the presence of an H_2O_2-generating system and labeled uric acid in phosphate buffer, yields among the oxidation products 5-ureido-2-imidazolidone-4,5-diol-4-carboxylic acid (UIDC, Formula II, Fig. 1), alloxanic acid, and a radioactive compound responsible for Peak A (Table II). Similar results have been previously obtained (18) from experiments in which borate buffer was present in a uricase system. The radioactive UIDC has the same properties as UIDC obtained from the uricase-borate system. It decomposes to C^{14}-labeled urea when obtained from an incubation mixture containing uric acid-8-C^{14}, and to C^{14}-labeled alloxanic acid when obtained from an incubation mixture containing uric acid-2-C^{14}. These results have been previously summarized (18).

It will be noted that an acidic component is present in the lactoperoxidase system, accounting for about 40 per cent of the total radioactivity. This acidic component can be eluted from the Dowex 1 column with concentrated formic acid. Initial attempts at purification have indicated that more than one component is present.

Verdoperoxidase and Horseradish Peroxidase—When C^{14}-uric acid is

TABLE I

*Oxidation Products of Uric Acid-2-C^{14} and Uric Acid-8-C^{14} by Catalase-Ethyl Hydrogen Peroxide System**

	URIC ACID 2-C^{14}	URIC ACID 8-C^{14}
UREA	6	5
ALLANTOIN	5	3
OXALYL DIUREA	5	5
CARBONYL DIUREA	23	16
CYANURIC ACID	24	21
PARABANIC ACID	0	30
OXALURIC ACID	0	10
OXONIC ACID 	20	10
CO_2	10	2

* Values expressed as per cent of the total eluted radioactivity.

incubated with verdoperoxidase or horseradish peroxidase, the end-products consist only of urea and allantoin (Table III). This finding is similar to the results obtained with uricase in the presence of phosphate buffer (18). In the presence of borate buffer, the verdoperoxidase system produces alloxanic acid.

Leucocyte suspensions prepared from sterile abscesses in rats behave like the verdoperoxidase system in phosphate buffer in that they oxidize uric acid to urea and allantoin (unpublished experiments).

When C^{14}-allantoin, isolated from the enzymatic decomposition of uric acid, was incubated in the presence of verdoperoxidase and notatin under

TABLE II

*Oxidation Products of Uric Acid-2-C^{14} and Uric Acid-8-C^{14} in Lactoperoxidase-H_2O_2 System**

	Urea	Allantoin	Peak A	Peak B	Alloxanic acid	Acid component
Uric acid-2-C^{14}	23	15	2	3	20	38
" acid-8-C^{14}	25	18	1	3	15	40

* Values expressed as per cent of the total eluted radioactivity.

TABLE III

*Oxidation Products of Uric Acid-2-C^{14} and Uric Acid-8-C^{14} by Verdoperoxidase-H_2O_2 and Horseradish Peroxidase-H_2O_2 Systems**

	Verdoperoxidase		Horseradish peroxidase	
	Urea	Allantoin	Urea	Allantoin
Uric acid-2-C^{14}	15	82	5	93
" acid-8-C^{14}	30	65		

* Values expressed as per cent of total eluted radioactivity.

conditions similar to those already described, no decomposition of allantoin was noted.

DISCUSSION

The peroxidases, verdoperoxidase and horseradish peroxidase, seem to attack uric acid in a manner qualitatively similar to that of uricase in phosphate buffer, although quantitative differences may exist. Here, as in the uricase-phosphate system, the only major end-products are urea and allantoin. The lactoperoxidase system which contains borate shows deviations from the other peroxidases similar to those obtained by the addi-

XUM

tion of borate to the uricase system. Alloxanic acid and UIDC are again apparent. In contrast to the uricase-borate system, an appreciable per cent of the radioactivity can be accounted for in the form of unidentified acidic components in the lactoperoxidase system.

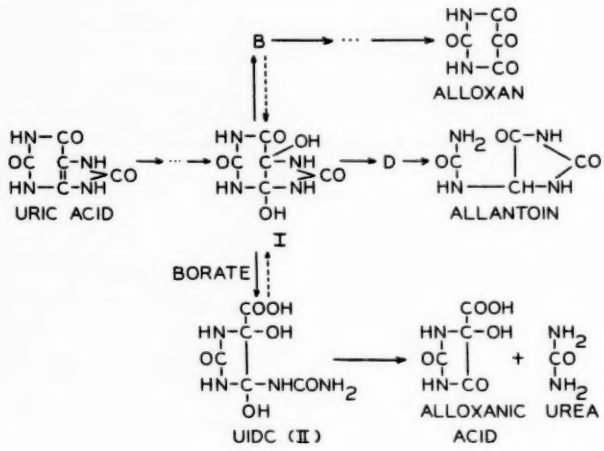


FIG. 1

The catalase-ethyl hydrogen peroxide system seems to represent a very much stronger peroxidative system than any of the others studied. While urea and allantoin are formed, a large number of allantoin oxidation products are also produced. In this system, in which the oxidation of uric acid proceeds to such an extent as to form a large number of end-products, including CO_2, the relative amounts of compounds formed have little meaning, since these are bound to change with the time of incubation and the amount of EtOOH available. The major difference between this system and the other peroxidases is that with the catalase-ethyl hydrogen peroxide system more than 1 atom of oxygen appears to be effectively utilized for the oxidation of uric acid, while with the other peroxidase systems, by forming end-products similar to those of uricase, only 1 atom of oxygen appears to be used effectively, in spite of the fact that about 10 atoms are available.

The biological significance of these results is at present difficult to evaluate. Folin *et al.* (3), in their review on uricolysis, had reached the conclusion that a non-dialyzable blood component and a small molecular weight tissue component react in the blood to decompose uric acid. In terms of the present discussion, these components could be thought of as a peroxidase and H_2O_2. Our results indicate that allantoin is a major end-

product of uric acid oxidation in a peroxidase-H_2O_2 system. In contrast, the experiments *in vivo* of Wyngaarden and Stetten show that, when N^{15}-uric acid is administered to humans, the major non-uric acid N^{15} component in the urine is urea. If peroxidases are responsible for the oxidation of uric acid in humans, then systems must be available to carry out the further oxidation of allantoin, since peroxidases in the presence of H_2O_2 will not decompose allantoin as previously mentioned. It is possible that *in vivo* the oxidation of uric acid goes beyond the allantoin stage, or that a catalase-peroxide system is responsible for the further oxidation of the latter.

An additional uricolytic system to be considered in organisms lacking uricase is cytochrome oxidase. Griffiths (21) has found that uric acid is oxidized by a crude beef heart cytochrome oxidase system at pH 7.9.

The results on the uric acid oxidation at neutral pH seem to exclude the possibility of alloxan being a precursor of alloxanic acid under our conditions. Rather, they indicate UIDC as a more direct precursor. Paul and Avi-Dor (22) have recently postulated the existence of an intermediate formed during the oxidation of uric acid by horseradish peroxidase and H_2O_2 at pH 3 to 5. This is converted to alloxan at pH values <1 or to allantoin at pH 6 to 7. The glycol structure which they propose (Formula I, Fig. 1) is compatible with its being a precursor of UIDC (Formula II). These results indicate that the scheme for uric acid oxidation which these authors propose may be modified as indicated in Fig. 1 in which D would represent the "allantoin precursor" found in the uricase system.

SUMMARY

1. The oxidative decomposition of uric acid-2-C^{14} and uric acid-8-C^{14} by a catalase-ethyl hydrogen peroxide system and by systems of lactoperoxidase-H_2O_2, verdoperoxidase-H_2O_2, and horseradish peroxidase-H_2O_2 has been studied at neutral pH.

2. The catalase-ethyl hydrogen peroxide system oxidizes uric acid beyond the allantoin stage. Among the labeled end-products of uric acid-2-C^{14} oxidation found are urea, allantoin, carbonyl diurea, oxalyl diurea, cyanuric acid, oxonic acid, and CO_2. Uric acid-8-C^{14} yields in addition labeled parabanic acid and oxaluric acid.

3. The lactoperoxidase-H_2O_2-borate system yields urea, allantoin, UIDC, and alloxanic acid in contrast to the verdoperoxidase-H_2O_2-phosphate and horseradish peroxidase-H_2O_2-phosphate systems, which yield only urea and allantoin.

4. The relationship of these systems, their possible biological significance, and some aspects of the mechanism of uric acid oxidation are discussed.

BIBLIOGRAPHY

1. Wiechowski, W., *Arch. exp. Path. u. Pharmakol.*, **60,** 185 (1909).
2. Umber, F., and Retzlaff, K., *Verhandl. Kong. inn. Med.*, **27,** 436 (1910).
3. Folin, O., Berglund, H., and Derick, C., *J. Biol. Chem.*, **60,** 361 (1924).
4. Benedict, J. D., Forsham, P. H., and Stetten, D., Jr., *J. Biol. Chem.*, **181,** 183 (1949).
5. Wyngaarden, J. B., and Stetten, D., Jr., *J. Biol. Chem.*, **203,** 9 (1953).
6. Canellakis, E. S., and Cohen, P. P., *J. Biol. Chem.*, **213,** 379 (1955).
7. Polis, B. D., and Shmukler, H. W., *J. Biol. Chem.*, **201,** 475 (1953).
8. Schultz, J., Shay, H., and Gruenstein, M., *Cancer Res.*, **14,** 157 (1954).
9. Tauber, H., and Petit, E. L., *J. Biol. Chem.*, **195,** 703 (1952).
10. Bonnichsen, R. K., Chance, B., and Theorell, H., *Acta chem. Scand.*, **1,** 685 (1947).
11. Kenten, R. H., and Mann, P. J. G., *Biochem. J.*, **57,** 347 (1954).
12. Baeyer, A., and Villiger, V., *Ber. chem. Ges.*, **34,** 738 (1901).
13. Biltz, H., and Topp, E., *Ber. chem. Ges.*, **46,** 1404 (1913).
14. Ohta, K., *Biochem. Z.*, **54,** 442 (1913).
15. Asche, A., *Ann. Chem.*, **416,** 226 (1918).
16. Menschutkin, N., *Ann. Chem.*, **172,** 73 (1874).
17. Wöhler, F., and Liebig, J., *Ann. Chem.*, **26,** 241 (1838).
18. Canellakis, E. S., and Cohen, P. P., *J. Biol. Chem.*, **213,** 385 (1955).
19. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **119,** 141 (1936).
20. Praetorius, E., *Biochim. et biophys. acta*, **2,** 602 (1948).
21. Griffiths, M., *J. Biol. Chem.*, **197,** 399 (1952).
22. Paul, K. G., and Avi-Dor, Y., *Acta chem. Scand.*, **8,** 637 (1954).

THE DISTRIBUTION OF ACONITASE AND FUMARASE IN HOMOGENATES OF HUMAN LIVER

By JAMES A. SHEPHERD, YAO WEN LI, EDWARD E. MASON, AND SIDNEY E. ZIFFREN

(From the Departments of Neurology and Surgery, State University of Iowa, College of Medicine, Iowa City, Iowa)

(Received for publication, August 10, 1954)

Earlier workers have demonstrated that "washed residue" fractions from homogenates of a variety of tissues contain the enzymes necessary to catalyze the complete oxidation of all of the known substrates of the tricarboxylic acid cycle (1, 2). When Hogeboom and Schneider (3) showed that 82 per cent of the isocitric dehydrogenase of C3H mouse liver homogenates was associated with the soluble fraction, it became necessary to reassess the conclusions drawn with respect to the distribution of other enzymes of the cycle. Aconitase has been reported to be associated with a particulate fraction of rabbit brain (4) and a non-particulate fraction of yeast (5). Evidence has subsequently been presented that the latter activity may have been derived from a particulate fraction (6). Aconitase has been reported to be associated with both a particulate and a non-particulate fraction of rabbit liver (7) and fumarase with rabbit brain (4) and mouse liver (8) mitochondria.

The evidence presented here indicates that the fumarase and aconitase activities in homogenates of human liver are associated largely with the supernatant fraction.

EXPERIMENTAL

Tissue Preparation—Specimens of human liver were obtained in the operating room and immediately chilled to 0°.[1] 2 gm. or less of liver were weighed and homogenized with 9 volumes of 0.25 M sucrose solution to prepare a 10 per cent homogenate. Since human liver contains a large amount of connective tissue, it was homogenized for 1 to 2 minutes in a very loose Potter-Elvehjem Pyrex homogenizer, filtered through a double layer of cheese-cloth, and rehomogenized with a well fitting homogenizer for 1 minute. An occasional whole or partially disintegrated cell could be found microscopically when several low power fields were examined.

[1] Each specimen of human liver was taken at the beginning of a scheduled operative procedure. Most of the patients were elderly and were being operated upon for duodenal or gastric ulcer or for gallbladder disease. None of the patients were jaundiced. Premedication included Nembutal, morphine, and scopolamine. Anesthesia was produced by cyclopropane, nitrous oxide and Pentothal sodium, or ether.

TABLE I

Distribution of Fumarase and Aconitase in Homogenates of Human Liver

	Enzyme activity*	Recovery of enzyme†	Total nitrogen*	Specific activity	Relative enzyme activity‡
Fumarase (4 fractionations)§					
	units per ml. ‖	*per cent*	*mg.*	*units per mg. N*	*per cent*
Original homogenate	2050	100	2.58	795	100
Sediment	454	22.2	1.35	336	42.3
Supernatant	1750	85.4	1.37	1227	154.3
Total recovery, %¶		107.6	105.4		
Aconitase (4 fractionations)**					
Original homogenate	348	100	2.58	134.9	100
Sediment	132	38.0	1.35	97.8	72.5
Supernatant	234	67.2	1.37	170.8	126.6
Total recovery, %¶		105.2	105.4		
Aconitase (3 fractionations)††					
Original homogenate	597	100	2.95	202.4	100
Sediment	158	26.5	1.55	101.9	50.3
Supernatant	467	78.2	1.58	295.6	146.1
Total recovery, %¶		104.7	106.1		

* Per ml. of original (10 per cent) homogenate or its equivalent.

† Activity of fraction × 100 divided by activity of original homogenate.

‡ Units per mg. of N of fraction × 100 divided by units per mg. of N of original homogenate.

§ The reaction mixtures contained 1.5 ml. of 0.1 M phosphate buffer, pH 7.4, tissue, and 0.3 ml. of 0.5 M sodium L-malate in a final volume of 3.0 ml. The blanks contained all additions except the substrate.

‖ 1 unit is defined as that amount of enzyme giving an increase in optical density of 0.001 per minute in the standard system.

¶ The average deviations of all the figures for "Total recovery, per cent" ranged from ±1.1 to ±3.6 per cent.

** The conditions were the same as for fumarase except that the substrate was 0.3 ml. of 0.3 M sodium citrate.

†† The conditions were the same as for fumarase except that the substrate was 0.2 l. of 0.1 M potassium DL-isocitrate.

Fractionation—5 ml. of 10 per cent homogenate were centrifuged at 25,000 × *g* for 30 minutes to sediment particulate components (an International model PR-1 refrigerated centrifuge was used). The sediment was washed once with 0.25 M sucrose at the same speed for the same length of time as was the original homogenate during the initial separation. The washed sediment was made up to a volume of 10 ml. and termed the sediment fraction. The combined supernatant fluid and washings were made to a volume of 10 ml. and designated the supernatant fraction. An aliquot of original 10 per cent homogenate was diluted to 5 per cent and called the original homogenate.

Enzyme Determinations—Fumarase and aconitase were determined by the method of Racker (9). Unless otherwise designated, the fumarase reaction mixtures contained 1.5 ml. of 0.1 M phosphate buffer, pH 7.4, 0.2 to 0.8 ml. of suitably diluted tissue suspension, 0.3 ml. of 0.5 M sodium L-malate, and 0.25 M sucrose solution to a final volume of 3.0 ml. The aconitase reaction mixtures were the same except that either 0.3 ml. of 0.3 M sodium citrate or 0.2 ml. of 0.1 M potassium DL-isocitrate was used as substrate. The blanks contained all additions except the substrate. All determinations were carried out at two or more concentrations of enzymes.

The reactions were initiated by addition of the enzyme. The progress of the reaction was followed at 25° in the Beckman DU spectrophotometer at 240 mμ with silica cells of 1.0 cm. light path. In each case the reaction was zero order, and the rate of the reaction was proportional to the concentration of the tissue preparations.

Materials and Chemical Determinations—The total nitrogen method and the preparation of potassium DL-isocitrate were described previously (4). The sodium L-malate and sodium citrate used were reprecipitated commercial samples.

Results

Table I shows that fumarase and aconitase (measured both from citrate to aconitate and from isocitrate to aconitate) are largely associated with the supernatant fraction of human liver homogenates. These enzymes are concentrated in this fraction. It may be seen also that the total recovery of enzyme closely parallels the total recovery of nitrogen.

SUMMARY

Human liver homogenates were prepared in 0.25 M sucrose and separated into sediment and supernatant fractions by centrifugation at 25,000 × *g* for 30 minutes.

Fumarase and aconitase were estimated by a rapid and sensitive spectrophotometric method. Each enzyme was largely associated with the super-

natant fraction of homogenates of human liver and was concentrated in that fraction.

BIBLIOGRAPHY

1. Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, **172,** 389 (1948).
2. Ochoa, S., *J. Biol. Chem.*, **155,** 87 (1944).
3. Hogeboom, G. H., and Schneider, W. C., *J. Biol. Chem.*, **186,** 417 (1950).
4. Shepherd, J. A., and Kalnitsky, G., *J. Biol. Chem.*, **207,** 605 (1954).
5. Hirsch, H. M., *Biochim. et biophys. acta*, **9,** 674 (1952).
6. Nossal, P. M., *Biochim. et biophys. acta*, **11,** 596 (1953).
7. Dickman, S. R., and Speyer, J. F., *J. Biol. Chem.*, **206,** 67 (1954).
8. Kuff, E. L., *J. Biol. Chem.*, **207,** 361 (1954).
9. Racker, E., *Biochim. et biophys. acta*, **4,** 211 (1950).

THE PHENYLALANINE AND TYROSINE REQUIREMENTS OF THE RAT*

By MARVIN D. ARMSTRONG

(From the Laboratory for the Study of Hereditary and Metabolic Disorders and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City, Utah)

(Received for publication, September 13, 1954)

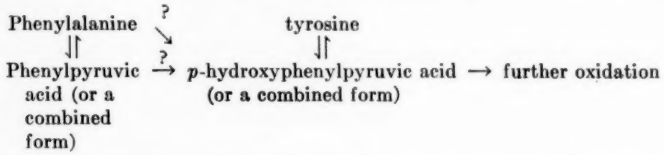
The work reported here is a continuation of experiments utilizing nutritional methods in an attempt to study the details of the oxidation of phenylalanine to tyrosine by the rat. Previous work on phenylalanine catabolism has indicated that conversion to tyrosine is quantitatively the most important and possibly the only pathway available for the oxidation of its aromatic nucleus. However, two lines of experimentation have produced evidence which has been interpreted as indicating that other pathways for the oxidation of the phenyl group of aromatic amino acids may exist. One concerns experiments on the metabolism of 4-methyl- and 4-methoxyphenylalanine by Dakin (2, 3), who reported that these derivatives are metabolized to CO_2 and H_2O by animals, but do not lead to an increase in homogentisic acid excretion when fed to alcaptonurics. Hence, they concluded that there is a pathway which does not involve the intermediate formation of homogentisic acid and suggested that phenylalanine also might be degraded by this alternative pathway. These experiments are not touched upon in this work, but will be considered in a later publication.

A different technique was used by Butts, Dunn, and Hallman (4) who studied the formation of glycogen and of ketone bodies from phenylalanine and tyrosine, respectively. They found that phenylalanine gave rise to a greater deposition of liver glycogen by fasted rats than was obtained with tyrosine. This caused them to doubt that phenylalanine is converted to glycogen by a pathway involving tyrosine as an intermediate, and they suggested that an alternative pathway might exist for the catabolism of phenylalanine. Other workers also have suggested that their experiments indicate that conversion to tyrosine may not be the primary reaction in the metabolism of phenylalanine (5).

An alternative suggestion which would explain these findings is that phenylalanine is not oxidized directly to tyrosine but to a derivative of

* This research was supported by a grant from the National Institutes of Health, United States Public Health Service. A preliminary report was presented at the meetings of the American Society of Biological Chemists, Chicago, April, 1953 (1).

tyrosine which can be converted to tyrosine or further oxidized without the intermediate formation of tyrosine. The first step in the oxidation of tyrosine in mammals has been shown to be transamination to form *p*-hydroxyphenylpyruvic acid (6); a possible scheme might be the following.



Although a previous experiment which was designed to test the possibility that phenylpyruvic acid might be an intermediate led to negative results (7) and, in addition, other work had indicated that phenylpyruvic acid probably was not involved as an intermediate (8), the following experiments were carried out in an attempt to gain more information which might prove or disprove the above formulation.

The growth experiments reported here establish the minimal optimal requirements for the growth of young rats under defined conditions for (1) L-phenylalanine in the absence of L-tyrosine, (2) L-phenylalanine in the presence of excess L-tyrosine, and (3) L-tyrosine in the presence of the minimal optimal amount of L-phenylalanine. In addition to the primary aim, which was to study the mechanism of oxidation of phenylalanine, this work provides data of nutritional interest because it presents a detailed study of the requirements for related essential and semiessential amino acids. Although it has been shown that tyrosine exerts a sparing action upon the dietary phenylalanine requirements (9) and that cystine likewise lowers the methionine requirement (10) and approximate optimal values have been reported in each case, a detailed study such as this has not been reported heretofore.

Materials and Methods

Young male albino rats (Sprague-Dawley) were used; for these experiments the conditions and basal diets were the same as those reported previously (7). The basal amino acid mixture was composed of amino acids obtained from commercial sources; this mixture was tested for phenylalanine with the method of Kapeller-Adler (11) and for tyrosine by a modification of Millon's test (12) and was found to be free of detectable amounts of either phenylalanine or tyrosine (<0.02 per cent). Recrystallized L-phenylalanine and L-tyrosine were added to the diets in the amounts indicated in Tables I, II, and III, and an equal weight of sucrose was omitted.

Rats weighing 50 to 55 gm. were used for all of these experiments; if the

initial weights were as low as 45 gm. or as high as 60 gm., the growth obtained on good diets was not consistent with the values observed under these conditions. An initial "depletion" period of 2 days was employed for all the animals; the advantages gained by this preliminary period have been commented upon previously (7). The weight changes reported here are for a period of 15 days following the end of the depletion period.

The results are reported as the average gain of the rats during the 15 day period; the standard deviations are somewhat greater than are desirable in experiments of this nature. This is because the experiments reported here were carried out over a period greater than a year with different lots of constituents for the basal diets, many different groups of young animals, and slightly different environmental conditions. Occasional inexplicable differences in the growth of different groups of rats under presumably the same conditions were observed, but, in order not to prejudice the results, no animals were discarded from the total of 170 used in this investigation with the exception of four which showed obvious signs of respiratory infection. It should be noted also that the conclusions drawn from these data are considerably more accurate than the standard errors of each level would appear to indicate, since the value obtained at each level is supported by the values for the levels on either side.

DISCUSSION

The first experiments which were carried out established the minimal amount of L-phenylalanine which can support optimal growth in the absence of tyrosine. The results, presented in Table I, show that 1.2 per cent in the diet is necessary. The earlier work of Rose and Womack (13) had indicated that 0.9 per cent of either L- or DL-phenylalanine was sufficient for optimal growth. The differences between the value obtained here and in the previous work are significant, as can be judged by the finding that rats receiving 1.2 per cent phenylalanine in these experiments gained 50 per cent more weight than those receiving 0.9 per cent phenylalanine and grew approximately 50 per cent faster than the animals of Rose and Womack. This difference did not arise because of the shorter growth periods used in these studies; actually, somewhat better average daily growth occurred in some experiments in which 25 day growth studies were made (7). An explanation of the difference probably lies in the better basal diets used here. The effect of better basal diets in modifying older results has been commented upon by Rose, Oesterling, and Womack (14) and in a previous publication from this laboratory (7).

The next set of experiments established the minimal dietary level of phenylalanine necessary to allow optimal growth in the presence of an excess of tyrosine. The results, presented in Table II, show that 0.6 per

cent is sufficient. This is in agreement with the previous results of Womack and Rose (9), who found that about 50 per cent of the necessary

TABLE I

Effect of L-*Phenylalanine on Growth in Absence of Tyrosine*

L-Phenylalanine added to basal diet	No. of rats	Average weight change	Average food consumption
per cent		*gm.*	*gm.*
None	2	−6.5 ± 0.5	29 ± 3.0
0.1	3	−3.7 ± 0.9	29 ± 2.5
0.2	3	−4.3 ± 0.5	35 ± 3.8
0.3	3	+1.0 ± 1.4	36 ± 5.5
0.4	3	+2.7 ± 1.7	38 ± 0.8
0.5	3	+6.3 ± 0.5	44 ± 1.9
0.6	6	+10.8 ± 1.5	49 ± 4.2
0.7	4	+18.8 ± 1.9	54 ± 3.5
0.8	5	+27.4 ± 2.0	66 ± 6.8
0.9	6	+30.0 ± 7.0	68 ± 4.8
1.0	6	+36.3 ± 6.8	71 ± 7.7
1.1	6	+40.0 ± 5.2	77 ± 7.8
1.2	8	+44.5 ± 7.5	81 ± 7.7
1.3	5	+46.2 ± 5.1	83 ± 7.7
1.4	6	+45.5 ± 5.6	81 ± 6.5
1.5	5	+48.0 ± 6.6	84 ± 6.0
1.6	5	+51.8 ± 7.3	90 ± 6.8

TABLE II

Effect of L-*Phenylalanine on Growth in Presence of Tyrosine*

L-Phenylalanine added to basal diet + 1.0 L-tyrosine	No. of rats	Average weight change	Average food consumption
per cent		*gm.*	*gm.*
None	2	−1.5 ± 0.5	30 ± 0
0.1	2	+4.5 ± 0.5	32 ± 1.0
0.2	2	+8.5 ± 2.5	48 ± 7.5
0.3	2	+19.5 ± 1.5	56 ± 3.5
0.4	3	+30.3 ± 2.9	67 ± 5.2
0.5	6	+41.0 ± 6.9	78 ± 9.6
0.6	7	+50.1 ± 4.8	90 ± 10.1
0.7	5	+46.6 ± 4.0	82 ± 3.9
0.8	5	+47.6 ± 3.5	83 ± 4.1
0.9	5	+47.8 ± 4.5	86 ± 7.0
1.0	7	+47.3 ± 6.0	81 ± 9.4

dietary phenylalanine could be replaced by tyrosine. Again, however, considerably better growth was observed in the present experiments.

The final set of experiments was designed to test the suggestion concerning the oxidation of phenylalanine to tyrosine. If an intermediate for the oxidation of phenylalanine to tyrosine also was an intermediate for the further oxidation of tyrosine, it might be expected that the oxidation to tyrosine would be inefficient, since some of the intermediate might be oxidized further rather than converted quantitatively to tyrosine. The efficiency of the conversion was tested in the following manner: With a diet containing an excess of tyrosine, only 0.6 per cent phenylalanine is required for optimal growth, and, in the absence of tyrosine, 1.2 per cent is required. If the conversion to tyrosine was quantitative, it could be

TABLE III

Effect of L-*Tyrosine on Growth in Presence of Minimal Optimal Amount of Phenylalanine*

L-Tyrosine added to basal diet + 0.6 L-phenylalanine	No. of rats	Average weight change	Average food consumption
per cent		*gm.*	*gm.*
None	6	+10.8 ± 1.5	49 ± 4.2
0.1	2	+23.5 ± 0.5	68 ± 1.0
0.2	3	+37.3 ± 0.9	76 ± 2.9
0.3	8	+42.5 ± 7.4	83 ± 4.8
0.4	7	+47.6 ± 5.1	87 ± 5.6
0.5	7	+45.4 ± 4.2	86 ± 4.6
0.6	6	+46.5 ± 8.0	82 ± 11.0
0.7	4	+50.2 ± 5.0	88 ± 7.2
0.8	4	+51.0 ± 5.0	86 ± 6.3
1.0	7	+50.1 ± 4.8	90 ± 10.1

estimated that 0.6 per cent of the dietary phenylalanine served to provide the minimal phenylalanine requirement and that 0.6 per cent was required for the formation of tyrosine; this would correspond to 0.7 per cent tyrosine in the diet. With a basal diet containing 0.6 per cent phenylalanine, however, it was found experimentally (Table III) that only 0.3 to 0.4 per cent tyrosine is adequate for optimal growth and the growth observed equals that obtained with 1.0 per cent tyrosine. The finding that only 50 per cent of the amount of phenylalanine in excess of that required to fulfil the phenylalanine requirement became available as tyrosine for growth indicates an inefficient conversion to tyrosine and is opposed to the hypothesis that phenylalanine is metabolized only by first being converted to tyrosine. It is, however, consistent with the proposed scheme whereby phenylalanine is converted to a derivative of tyrosine, which either may form tyrosine or be further oxidized.

SUMMARY

1. The minimal optimal dietary requirements for the young albino rat under defined conditions for L-phenylalanine in the absence of L-tyrosine, for L-phenylalanine in the presence of excess L-tyrosine, and for L-tyrosine in the presence of the minimal optimal amount of L-phenylalanine have been established. In the absence of tyrosine, 1.2 per cent of L-phenylalanine is necessary; in the presence of excess tyrosine, 0.6 per cent L-phenylalanine is necessary; and in the presence of 0.6 per cent L-phenylalanine, between 0.3 and 0.4 per cent L-tyrosine is necessary.

2. The finding that there is inefficient conversion of phenylalanine to tyrosine supports the hypothesis that an intermediate in the oxidation of phenylalanine to tyrosine may also be an intermediate for the further oxidation of tyrosine.

BIBLIOGRAPHY

1. Armstrong, M. D., *Federation Proc.*, **12,** 171 (1953).
2. Wakeman, A. J., and Dakin, H. D., *J. Biol. Chem.*, **9,** 139 (1911).
3. Dakin, H. D., *J. Biol. Chem.*, **9,** 151 (1911).
4. Butts, J. S., Dunn, M. S., and Hallman, L. F., *J. Biol. Chem.*, **123,** 711 (1938).
5. Shambaugh, N. F., Lewis, H. B., and Tourtellotte, D., *J. Biol. Chem.*, **92,** 499 (1931).
6. Knox, W. E., and LeMay-Knox, M., *Biochem. J.*, **49,** 686 (1951).
7. Armstrong, M. D., *J. Biol. Chem.*, **205,** 839 (1953).
8. Udenfriend, S., and Cooper, J. R., *J. Biol. Chem.*, **194,** 503 (1952).
9. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **166,** 429 (1946).
10. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **141,** 375 (1941).
11. Kapeller-Adler, R., *Biochem. Z.*, **252,** 185 (1932).
12. Bernhart, F. W., and Schneider, R. W., *Am. J. Med. Sc.*, **205,** 636 (1943).
13. Rose, W. C., and Womack, M., *J. Biol. Chem.*, **166,** 103 (1946).
14. Rose, W. C., Oesterling, M. J., and Womack, M., *J. Biol. Chem.*, **176,** 753 (1948).

THE CHEMICAL NATURE OF THE FATTY ACIDS OF A GROUP C STREPTOCOCCUS SPECIES*

By KLAUS HOFMANN and FRED TAUSIG

(*From the Biochemistry Department, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania*)

(Received for publication, September 22, 1954)

The mechanism of stimulation of bacterial growth by lipides is as yet but little understood. Since it seemed to us that exact knowledge of the fatty acid composition of a number of microorganisms might provide a basis for further advances in this field, we have recently examined the fatty acid spectra of *Lactobacillus arabinosus* (1, 2) and of *Lactobacillus casei* (3). It was found that the fatty acid patterns of these organisms are practically identical; both lactobacilli produce palmitic, stearic, *cis*-vaccenic, and lactobacillic acids as the major lipide components.

The present investigation extends our fatty acid studies to a group C *Streptococcus* species.

EXPERIMENTAL[1]

Cultivation and Extraction of Cells

The starting material for this study was a large quantity of cells of a group C *Streptococcus* species, strain H46A (ATCC 10706), grown on Christensen's medium (4). At the end of the incubation period, a quantity of Hyflo Super-Cel was added to the culture to facilitate collection of the cells and the mixture filtered; the moist filter cake (5340 gm.), composed of streptococcal cells and Hyflo Super-Cel (inactivated by the addition of *n*-hexylresorcinol), was then shipped to our laboratory.[2] Immediately on arrival, this material was suspended in 14 liters of acetone and the suspension was kept at 5° for 48 hours. The acetone extract was then decanted from the insoluble residues which were reextracted with six additional 5 liter portions of acetone. The combined acetone extracts were evaporated

* Supported by grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, the Rockefeller Foundation in New York, and Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

[1] The melting points were determined with short stem Anschütz thermometers and are uncorrected. Freshly distilled solvents were used, and operations were performed under nitrogen whenever necessary. The iodine numbers were determined by the Wijs method.

[2] We wish to express our sincere appreciation to Dr. Brian Hutchings of the Lederle Laboratories Division of the American Cyanamid Company for supplying us with this material.

to a small volume *in vacuo*, and the resulting suspension was extracted with ether. The ether extract was washed with 5 per cent sodium bicarbonate, dried over sodium sulfate, and evaporated to give a yellow oil (12 gm.) composed of "free lipides" and *n*-hexylresorcinol. The material insoluble in acetone was collected and dried to constant weight *in vacuo*. The dry residue weighed 3420 gm. and contained 430 gm. of combustible substance (streptococcal cells) as determined by ashing of a small aliquot. For the isolation of the "bound lipides" this residue was suspended in 12.5 liters of 2 N sulfuric acid and the mixture was autoclaved at 121° for 2 hours. The cooled hydrolysate was filtered through large Büchner funnels and the filter cakes were exhaustively extracted with ether. The combined ether extracts were washed with 5 per cent sodium bicarbonate, dried over sodium sulfate, and evaporated *in vacuo* to give 15.1 gm. of "bound lipides." The filtrate was discarded, as exhaustive extraction of a 300 ml. aliquot yielded only a negligible quantity (13 mg.) of ether-soluble material.

Isolation and Characterization of Fatty Acids from "Bound Lipides"

Preparation and Separation of Methyl Esters

Saponification of the "bound lipides" (15.1 gm.) (2) gave 13.6 gm. of tan-colored fatty acids (iodine number 36.6) and 0.39 gm. of a light brown, semisolid, non-saponifiable fraction which was not investigated further. A microspectrophotometric analysis (5) of the fatty acids demonstrated the presence of 2.8 per cent of dienoic acids, 0.2 per cent of trienoic acids, and 0.0 per cent of tetraenoic acids. A sample of the fatty acids (12.6 gm.) was esterified with diazomethane and the methyl esters (12.93 gm.) were fractionally distilled in a spinning band, semimicro, Piros Glover fractionating column (2). From the distillation curve (Fig. 1), the following composition was estimated: C_{12} esters, 0.26 gm. (2.0 per cent); C_{14} esters, 0.67 gm. (5.2 per cent); C_{16} esters, 4.85 gm. (37.5 per cent); C_{18} esters, 5.62 gm. (43.4 per cent); and still pot residues 1.54 gm. (11.9 per cent).

C_{12} Fatty Acid

The C_{12} methyl ester Fractions[3] 1 and 2 (0.215 gm.) melted at 3.2–4.8° and gave no depression of melting point on admixture with methyl laurate. Saponification followed by recrystallization from acetone afforded lauric acid, m.p. 43.8–44.8°; neutral equivalent, calculated, 200; found, 204.

C_{14} Fatty Acid

The C_{14} methyl ester Fractions 5 and 6 (0.356 gm.) melted at 17.3–17.8° and gave no depression of melting point on admixture with methyl my-

[3] The individual fractions collected during the course of the distillation were numbered consecutively and individual points on the distillation curve (Fig. 1), from left to right, correspond to fraction numbers.

ristate. Saponification and recrystallization from acetone afforded myristic acid, m.p. 54.2–54.8°; neutral equivalent, calculated, 228; found, 228.

C_{16} Fatty Acids

The C_{16} methyl ester Fractions 19 and 20 (1.192 gm.) melted at 29.0–30.0° and gave no depression of melting point on admixture with methyl palmitate. Saponification followed by recrystallization from acetone afforded palmitic acid, m.p. 63.0–63.5°; neutral equivalent, calculated, 256.4; found, 256. A sample of the acid was converted into the amide which melted at 105.0–106.1° and gave no depression on admixture with palmitamide. The C_{16} methyl ester Fractions 11 to 15 (1.855 gm.) were

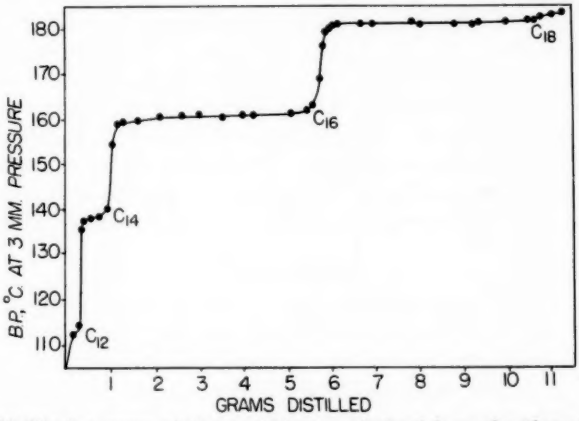


FIG. 1. Distillation curve of the methyl esters derived from the "bound lipides"

saponified and the resulting acids (1.770 gm.) were separated into palmitic acid, m.p. 61.8–63.2° (0.773 gm.), and a liquid acid fraction (0.978 gm.) by fractional recrystallization at −20° from petroleum ether (eight recrystallizations) and then from acetone (seven recrystallizations). The liquid acid fraction was purified further by low temperature recrystallization from acetone (three recrystallizations at −50° followed by five recrystallizations at −80°) to give 0.406 gm. of a material melting at −14° to −12°; iodine number, 99.8; neutral equivalent, 254. (Calculated, iodine number, 99.8; neutral equivalent, 254.)

A sample of this material (51.0 mg.) was subjected to microhydrogenation (6) and palmitic acid (36 mg.), m.p. 61.8–62.6°, was isolated from the hydrogenation products.

Performic acid oxidation (7) of a sample of the liquid material (103.0 mg.) gave a dihydroxyhexadecanoic acid fraction (108 mg.) which was recrystallized from aqueous ethanol (three recrystallizations from 50 per

cent ethanol at 5° and three recrystallizations from 95 per cent ethanol at −20°); m.p. 77.2–79.6°. (Literature, melting point of the low melting dihydroxy derivative of palmitoleic acid is 85–86° (8).)

$C_{16}H_{32}O_4$. Calculated, C 66.6, H 11.2; found, C 66.3, H 10.9

The dihydroxyhexadecanoic acid fraction (200 mg.) was oxidized with periodic acid (9) and the oxidation mixture was steam-distilled. The nonvolatile fragments (aldehydocarboxylic acids) were converted into the corresponding dibasic acids by oxidation with silver oxide in the presence of dilute sodium hydroxide in the manner previously described (2). The dibasic acids (75 mg.) melted at 83.6–90.8° and had a neutral equivalent of 98. Samples of these dibasic acids were subjected to chromatography on phosphate-buffered silica gel columns according to the method of Klenk and Bongard (10). Klenk's Column B served as the stationary phase and chloroform containing 5 per cent of *n*-butanol was employed for the elution of undecanedioic acid; azelaic acid was eluted with chloroform containing 30 per cent of *n*-butanol. A typical run, with an 8.6 mg. sample of the dibasic acids, is illustrated in Fig. 2. The azelaic acid fractions from two chromatograms were combined for isolation of the pure acid, m.p. 104.8–106.8°; there was no depression on admixture with an authentic sample.

C_{18} *Fatty Acids*

The C_{18} methyl ester Fractions 36, 37, and 39 (0.937 gm.), m.p. 36.5–38.0°, were saponified to give stearic acid, m.p. 68.9–69.8°; neutral equivalent, calculated, 284; found, 283. The C_{18} methyl ester Fractions 28 to 32 (2.370 gm.) were saponified, and a 1.950 gm. sample of the resulting acids was separated into stearic acid, m.p. 68.6–69.6° (0.630 gm.), and a liquid acid fraction (1.294 gm.) by low temperature recrystallization essentially in the manner previously described (3). Ten additional recrystallizations of the liquid acid fraction from acetone at −60° gave a material (0.694 gm.) having the following properties: m.p. 10.5–12.0°, iodine number, 89.7, neutral equivalent, 280; calculated, iodine number, 89.9; neutral equivalent, 282. No depression of the melting point was observed when this material was admixed with a sample of synthetic *cis*-vaccenic acid; a 1:1 mixture with oleic acid melted at 2.0–3.2°. The oxirane derivative of the liquid acid (1) melted at 46.0–47.2° and did not depress the melting point of a sample of the oxirane derivative of synthetic *cis*-vaccenic acid, m.p. 46.0–47.6°. On admixture with the oxirane derivative of oleic acid, m.p. 57.0–58.0°, a marked depression of the melting point was observed.

Performic acid oxidation (7) of a 99 mg. sample of the liquid acid afforded a dihydroxyoctadecanoic acid melting at 89.6–91.2°. This ma-

terial did not depress the melting point of an authentic sample of the low melting dihydroxy derivative of synthetic *cis*-vaccenic acid, but a marked depression resulted on admixture with the low melting dihydroxy derivative of oleic acid.

$C_{18}H_{36}O_4$. Calculated, C 68.3, H 11.5; found, C 68.1, H 11.3

A sample of the dihydroxy derivative (35 mg.) was oxidized with periodic acid followed by silver oxide, as previously described (2), and the resulting

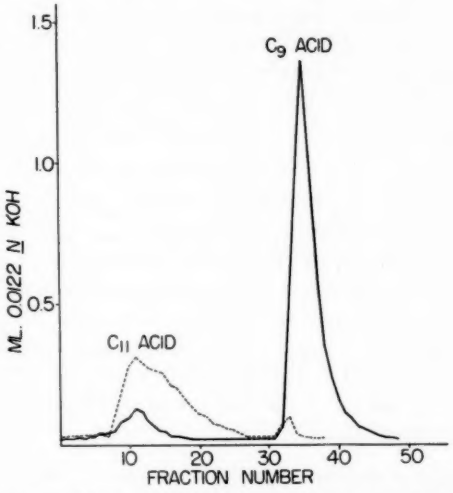


FIG. 2. Chromatographic analysis of dibasic cleavage products from the liquid C_{16} acid (solid curve) and the liquid C_{18} acid (dotted curve) derived from "bound lipides." Volume of individual fraction, 7 ml. C_9 acid = azelaic acid; C_{11} acid = undecanedioic acid.

dibasic acids were dissolved in 10 ml. of chloroform. Aliquots of this solution were chromatographed on Klenk's Column B with chloroform containing 5 and 30 per cent, respectively, of *n*-butanol serving as eluting agents. A typical run, with a 2 ml. aliquot of the chloroform solution of the dibasic acids, is illustrated in Fig. 2.

Examination of Still Pot Residues

The dark brown, viscous, still pot residues (1.542 gm.) were distilled at 0.05 mm. to give 0.894 gm. of pale yellow distillate and 0.640 gm. of a resinous material which was not investigated further. The distillate was saponified, and the resulting acids were separated into a solid fraction

(0.342 gm.) and a liquid fraction (0.466 gm.) by fractional recrystallization from acetone at −20°. Spectroscopic examination of the liquid fraction in the infra-red region failed to reveal the presence of lactobacillic acid.

Isolation and Characterization of Fatty Acids from "Free Lipides"[4]

The substances extractable with acetone (12.0 gm.) consisting of *n*-hexylresorcinol and "free lipides" were saponified to give an alkali-soluble fraction (*n*-hexylresorcinol plus fatty acids) (9.0 gm.) and alkali-insoluble materials (0.36 gm.) which were not investigated further. The alkali-soluble portion (8.783 gm.) was exposed to an excess of diazomethane for 10 minutes, and the resulting material (8.955 gm.) was distilled. This material was composed of *n*-hexylresorcinol, 2.924 gm.; low boiling esters, 0.256 gm.; C_{16} esters, 1.627 gm.; C_{18} esters, 1.607 gm.; and still pot residues, 2.541 gm.

C_{16} Fatty Acids

A sample of the C_{16} esters (1.300 gm.) was saponified, and the resulting acids (1.235 gm.) were separated into palmitic acid, m.p. 62.2–63.4° (0.700 gm.), and a liquid fraction (0.535 gm.) by fractional recrystallization from acetone at −30°. The liquid material was further purified by four recrystallizations from acetone at −80° to give a fraction (0.297 gm.) melting at −10° to −8°; neutral equivalent, 253; iodine number, 100.4.

The dihydroxy derivative melted at 79.0–83.0° and did not depress the melting point of a sample of the corresponding dihydroxy derivative derived from the "bound lipides."

$C_{16}H_{32}O_4$. Calculated, C 66.6, H 11.2; found, C 66.6, H 11.0

C_{18} Fatty Acids

A sample of the C_{18} methyl esters (1.179 gm.) was saponified and the resulting acids were separated into stearic acid, m.p. 67.8–69.2° (0.260 gm.), and a liquid fraction (0.858 gm.) by fractional recrystallization from acetone at −25°. The liquid material was further purified by six recrystallizations from acetone and two recrystallizations from methanol at −60° to give a fraction (0.413 gm.) melting at 9.0–11.0°; neutral equivalent, 281; iodine number, 89.6. Admixture with *cis*-vaccenic acid gave no lowering of the melting point, but a marked depression resulted on admixture with oleic acid.

The dihydroxy derivative melted at 90.8–92.4° and gave no depression when admixed with a sample of the corresponding dihydroxy derivative from the "bound lipides."

[4] The presence of large amounts of *n*-hexylresorcinol in the substances extractable with acetone interfered with the isolation of the "free lipide" fatty acids and the quantitative evaluation of the complete fatty acid spectrum.

DISCUSSION

As far as we are able to ascertain, the present investigation represents the first systematic study of the fatty acid spectrum of a *Streptococcus*. The total lipide content of the organisms (grown under the conditions specified) was 4.5 per cent, based on the weight of dry cells. The major portion of these lipides was present in a "bound" form, and only a small proportion of acetone-extractable "free lipides" was isolated. The "bound lipides" contained 2.8 per cent of dienoic acids: the proportion of tri- and tetraenoic acids was negligible. The "bound lipide" methyl esters consisted of large proportions of C_{16} and C_{18} esters and of small proportions of C_{12} and C_{14} esters. Pure samples of lauric and myristic acids were isolated from the lowest boiling ester fractions. The C_{16} esters contained both solid and liquid constituents. The solid acid was identified as palmitic acid; the neutral equivalent and iodine number of the highly purified liquid fraction were those of a monoethenoid hexadecanoic acid. Hydrogenation converted the material into palmitic acid, demonstrating the presence of a straight carbon chain. The dihydroxy derivative of the liquid fraction melted unsharply and considerably lower than the low melting isomer of 9,10-dihydroxyhexadecanoic acid. Its oxidative cleavage resulted in the formation of azelaic and undecanedioic acids in the ratio of 89:11 on a molar basis. We conclude that the liquid fraction contains large proportions of 9,10-hexadecenoic acid (palmitoleic acid) and smaller proportions of 11,12-hexadecenoic acid (palmitvaccenic acid). We are unaware of any previous report indicating the presence of the latter material in a natural source.

In addition to stearic acid, the C_{18} acids contained liquid constituents which exhibited the neutral equivalent and iodine number of a monoethenoid octadecanoic acid. The liquid material and its dihydroxy and oxirane derivatives failed to depress the melting point of *cis*-vaccenic acid and of its corresponding derivatives, respectively. A marked depression resulted with the corresponding compounds of the oleic acid series. These observations suggest the presence of *cis*-vaccenic acid contaminated by small quantities of a closely similar monoethenoid octadecanoic acid or acids. The x-ray analysis of the dihydroxy derivative also pointed to the presence of a mixture largely consisting of 11,12-dihydroxyoctadecanoic acid and a small proportion of a closely similar material, possibly 9,10-dihydroxyoctadecanoic acid.[5] The presence of undecanedioic and azelaic acids in the ratio of 9:1 on a molar basis in the dibasic cleavage fragments of this dihydroxy derivative leads to the final characterization of the liquid fraction as a mixture of *cis*-vaccenic and oleic acids.

[5] We wish to thank Dr. E. S. Lutton of The Procter and Gamble Company for the x-ray pattern and its evaluation.

Thus far, we have investigated the fatty acid patterns of *L. arabinosus* and *L. casei* (2, 3), and have shown that these organisms contain *cis*-vaccenic acid as the major monoethenoid octadecanoic acid component of their lipides. The characterization of the *cis*-vaccenic acid was based on rigorous physical and chemical criteria. The streptococcal lipides also contain large proportions of *cis*-vaccenic acid, with minor proportions of oleic acid. It is of interest to note that the double bond of the major monounsaturated C_{18} component (*cis*-vaccenic acid) of the streptococcal lipides occupies the 11,12 position, whereas the double bond of the major monoethenoid hexadecanoic acid (palmitoleic acid) is located in the 9,10 position.

Of particular significance is the absence of lactobacillic acid from the streptococcal lipides. This fatty acid is present in significant proportion in the lipides of the previously mentioned organisms. In our isolations of this cyclopropane fatty acid, we had resorted to diazomethane for conversion of the fatty acids into their methyl esters. Diazomethane has the property of adding across certain double bonds. This suggested the remote possibility that lactobacillic acid may be an artifact produced by the addition of diazomethane to the double bond of *cis*-vaccenic acid, which occurs in these lipides. In the present investigation we have again employed the diazomethane method of esterification and have not been able to detect lactobacillic acid, despite the presence of *cis*-vaccenic acid. Lactobacillic acid is thus a true metabolite and not an artifact produced during the isolation.

SUMMARY

The fatty acid spectrum of a Group C *Streptococcus* species, strain H46A, has been qualitatively evaluated. The lipide content of the dry cells was 4.5 per cent. The major portion of these lipides was present in a "bound" form, and only a minor fraction was acetone-extractable ("free lipides"). The "bound lipides" were composed of small proportions of lauric and myristic acids and large proportions of C_{16} and C_{18} fatty acids. The C_{16} fatty acid fraction contained palmitic, 9,10-hexadecenoic, and a small proportion of 11,12-hexadecenoic acids. In addition to stearic acid, the C_{18} fraction consisted of a large proportion of *cis*-vaccenic acid and small proportions of oleic acid. A careful investigation of the highest boiling ester fractions derived from the "bound lipides" failed to reveal the presence of lactobacillic acid. The composition of the "free lipides" was similar to that of the "bound lipides."

BIBLIOGRAPHY

1. Hofmann, K., and Lucas, R. A., *J. Am. Chem. Soc.*, **72,** 4328 (1950).
2. Hofmann, K., Lucas, R. A., and Sax, S. M., *J. Biol. Chem.*, **195,** 473 (1952).

3. Hofmann, K., and Sax, S. M., *J. Biol. Chem.*, **205,** 55 (1953).
4. Christensen, L. R., *J. Gen. Physiol.*, **28,** 363 (1945).
5. Report of the Spectroscopy Committee, *J. Am. Oil Chem. Soc.*, **26,** 399 (1949).
6. Johns, I. B., and Seiferle, E. J., *Ind. and Eng. Chem., Anal. Ed.*, **13,** 841 (1941).
7. Swern, D., Billen, G. N., Findley, T. W., and Scanlan, J. T., *J. Am. Chem. Soc.*, **67,** 1786 (1945).
8. Boughton, B. W., Bowman, R. E., and Ames, D. E., *J. Chem. Soc.*, 671 (1952).
9. Huber, W. F., *J. Am. Chem. Soc.*, **73,** 2730 (1951).
10. Klenk, E., and Bongard, W., *Z. physiol. Chem.*, **290,** 181 (1952).

ON THE IDENTITY OF PHYTOMONIC AND LACTOBACILLIC ACIDS. A REINVESTIGATION OF THE FATTY ACID SPECTRUM OF AGROBACTERIUM (PHYTOMONAS) TUMEFACIENS*

By KLAUS HOFMANN and FRED TAUSIG

(From the Biochemistry Department, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania)

(Received for publication, September 22, 1954)

The lipides of the plant pathogen *Agrobacterium* (*Phytomonas*) *tumefaciens* have been the subject of previous investigations. Chargaff and Levine (1) examined the lipide components of the organism, grown on a natural bean broth medium, and found them to contain glycerol, sterols, palmitic and oleic acids, and a complex mixture of higher unsaturated fatty acids. They were the first to recognize the occurrence in these lipides of a liquid-saturated acid, but made no attempt to explore the chemical nature of this material. Later, the lipides of *A. tumefaciens* were investigated by Anderson and his collaborators (2, 3). These workers stated that the composition of the culture medium exerts a pronounced effect on the lipide composition of the organisms, and they employed a synthetic medium containing sucrose and glutamic acid as the sole organic constituents for mass culturing of the bacteria. The acetone-soluble fat, derived from organisms grown on this medium, was found to contain a large proportion of unsaturated, and only a minor quantity of saturated, fatty acids. They concluded that the unsaturated fatty acid was oleic acid, since it was converted into stearic acid by catalytic hydrogenation. The presence of a liquid-saturated fatty acid in these lipides was confirmed, and this material was characterized as having the elementary formula $C_{20}H_{40}O_2$ (by neutral equivalent weight determinations and carbon-hydrogen analyses). The physical and chemical properties of this liquid-saturated acid were further explored by Velick (4, 5), who employed Anderson's hydrogenation technique for the separation of the liquid-saturated from the liquid-unsaturated acids. Velick's purest preparation melted at 24° and formed a hydrazide of melting point 56.6°. In view of its occurrence in *Phytomonas* cells, the name "phytomonic acid" was suggested for

* Supported by grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, the Rockefeller Foundation in New York, and Ciba Pharmaceutical Products, Inc., Summit, New Jersey. Presented in part at the Thirty-eighth annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, April, 1954.

the compound and the elementary formula previously assigned by Anderson was confirmed. Chemical degradation studies were not carried out, but the x-ray diffraction pattern of "phytomonic acid" was obtained; comparison with the diffraction patterns of a series of long chain fatty acids having straight or branched carbon chains indicated the structure of a 10- or 11-methylnonadecanoic acid for "phytomonic acid." Cavanna and Ställberg-Stenhagen (6) synthesized the *d* and *l* isomers of 11-methylnonadecanoic acid and found them to differ from "phytomonic acid."

Our studies on the lipides of *Lactobacillus arabinosus* (7) and of *Lactobacillus casei* (8) led to the discovery of a novel fatty acid of the formula $C_{19}H_{36}O_2$ which was shown to be a cyclopropane derivative (9). This lactobacillic acid melts at 28–29° and exhibits a long spacing value of 41.0 A. A comparison of the melting point (24°) and the long spacing value (40.5 A) of "phytomonic acid" with the corresponding constants of lactobacillic acid suggested to us the possibility that these compounds might be identical and prompted the present reevaluation of the fatty acid spectrum of *A. tumefaciens*. We find that "phytomonic" and lactobacillic acids are, indeed, identical.

EXPERIMENTAL[1]

Cultivation and Extraction of Cells

A large quantity of cells of *A.* (*Phytomonas*) *tumefaciens*, strain A6K1, was grown under aerobic conditions at 25° (at Lederle Laboratories). The medium (which corresponds to Medium 2 of Geiger and Anderson (2)) had the following composition: sucrose 10.0 gm., monosodium glutamate 2.9 gm., sodium chloride 0.2 gm., magnesium sulfate heptahydrate 0.2 gm., calcium chloride dihydrate 0.1 gm., dipotassium phosphate 1.0 gm., and ammonium sulfate 7.5 gm. in distilled water (1000 ml.) and was adjusted to pH 7.4 with sodium hydroxide. The cells were collected by centrifugation, and the ensuing wet suspension was frozen (weight 1483 gm.), packed in dry ice, and shipped to our laboratory. A small aliquot of the material was dried to constant weight over phosphorus pentoxide *in vacuo* for determination of its content of dry bacteria (345 gm.). For extraction of the lipides, the cells were thawed and then autoclaved in 3.5 liters of 2 N sulfuric acid at 121° for 1.5 hours. Hyflo Super-Cel (250 gm.) was added to the cooled hydrolysate, and the suspension was kept at 5° for 12 hours, when it was filtered through a 24 cm. Büchner funnel. The filter cake was

[1] The melting points were determined with short stem Anschütz thermometers and are uncorrected. Freshly distilled solvents were used, and operations were performed under nitrogen whenever necessary. The iodine numbers were performed by the Wijs method.

pressed as dry as possible and suspended in 2.5 liters of acetone, and the suspension was refluxed for 3 hours. The acetone extract was removed by decantation, and the residue was reextracted in a similar manner with three 2.5 liter portions of acetone and then 2.5 liter portions of ether. The combined acetone-ether extracts were concentrated to a small volume *in vacuo* and water (500 ml.) was added. The suspension was extracted with ether (four 1 liter portions), the combined ether extracts were washed with 5 per cent sodium bicarbonate, and dried over sodium sulfate, and the ether was removed *in vacuo* to give 25.85 gm. of crude lipides.

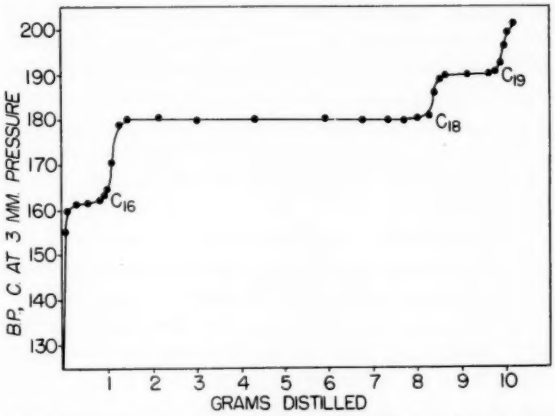


FIG. 1. Distillation curve of the methyl esters derived from *A. tumefaciens* lipides

Isolation and Characterization of Fatty Acids

Preparation and Separation of Methyl Esters

The lipides (25.85 gm.), on saponification, afforded 19.00 gm. of fatty acids and 2.84 gm. of non-saponifiable materials. The fatty acids were esterified with diazomethane to give 19.46 gm. of crude methyl esters. A mixture of the methyl esters (10.39 gm.) with a high boiling paraffin wax[2] (2.5 gm., which served as a "chaser") was distilled in a Piros Glover still. From the distillation curve (Fig. 1), it was estimated that the ester mixture contained C_{16} methyl esters, 1.06 gm. (10.2 per cent); C_{18} methyl esters, 7.07 gm. (68.6 per cent); C_{19} methyl esters, 1.36 gm. (13.1 per cent); and still pot residues, 0.90 gm. (8.6 per cent).[3]

[2] A paraffin wax fraction, b.p. 180–220° at 3 mm., was employed.

[3] The paraffin wax content of the distillate fractions was determined and subtracted to arrive at these figures.

C_{16} *Fatty Acid*

Distillate Fractions [4] 3 to 8 (0.972 gm.) were combined and saponified to give 0.873 gm. of fatty acids and 0.030 gm. of non-saponifiable material (paraffin wax). The fatty acid fraction, upon recrystallization from acetone, yielded 0.655 gm. of palmitic acid, m.p. 61.4–62.0°.

C_{18} *Fatty Acids*

Distillate Fractions 11 to 19 (6.901 gm.) were combined and saponified to give 6.206 gm. of fatty acids, m.p. 11.2–12.6°, and 0.248 gm. of non-saponifiable material (paraffin wax). A sample (1.503 gm.) of the fatty acids was recrystallized from acetone (three recrystallizations at −80°, three recrystallizations at −60°, and three recrystallizations at −25°) to yield 1.220 gm. of a material melting at 15.0–15.8° (iodine number 89.3). This substance gave no melting point depression on admixture with *cis*-vaccenic acid; with oleic acid, a marked depression of the melting point was observed. By our method of analysis, we were unable to detect stearic acid in the C_{18} fraction.

A sample of the liquid acid (0.200 gm.) was converted into its dihydroxy derivative by performic acid oxidation (10). Following recrystallization from 50 per cent aqueous ethanol at 0° (three recrystallizations) and from acetone at −20° (two recrystallizations), the dihydroxy derivative melted at 92.8–94.0°. No melting point depression resulted upon admixture with the low melting form of 11,12-dihydroxyoctadecanoic acid; with the low melting form of 9,10-dihydroxyoctadecanoic acid, a marked depression was observed. The x-ray diffraction pattern was that of the low melting form of 11,12-dihydroxyoctadecanoic acid.

$C_{18}H_{36}O_4$. Calculated, C 68.3, H 11.5; found, C 68.2, H 11.4

C_{19} *Fatty Acid*

Distillate Fractions 23 to 28 (1.389 gm.) were combined and, on saponification, gave 1.086 gm. of fatty acids and 0.184 gm. of non-saponifiable material (paraffin wax). Following recrystallization from acetone at −55° (five recrystallizations), the fatty acid (0.801 gm.) melted at 27.8–28.8° and gave no depression on admixture with lactobacillic acid. A comparison of the infra-red absorption spectrum of this material with that of lactobacillic acid from *L. arabinosus* is shown in Fig. 2. The long spacings and the main side spacings of the acid from *A. tumefaciens* are compared with those of lactobacillic acid from *L. arabinosus* in Table I.

[4] The individual fractions collected during the course of the distillation were numbered consecutively, and individual points on the distillation curve (Fig. 1), from left to right, correspond to fraction numbers.

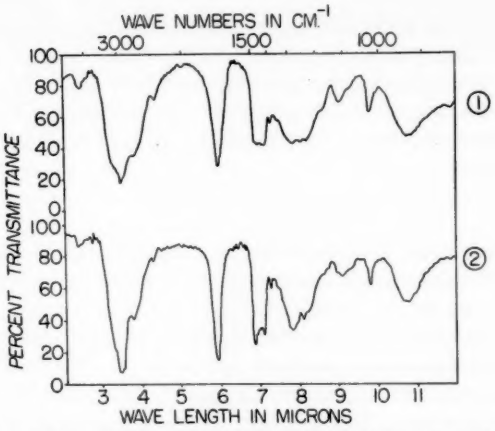


FIG. 2. Infra-red absorption curves of lactobacillic acid from *L. arabinosus* (Curve 1) and from *A. tumefaciens* (Curve 2), obtained by using a thin film of the compounds between rock salt prisms.

TABLE I

Comparison of Main x-Ray Spacings of Lactobacillic Acid from L. arabinosus and from A. tumefaciens

L. arabinosus	*A. tumefaciens*
Main short spacings	
4.65 M.	4.67 S.
4.35 "	4.38 M. 4.30 S.
4.07 M.−	4.07 W.+
3.78 W.	3.81 W.
3.58 W.+	3.61 "
3.42 W.−	3.43 W.−
Long spacing	
41.0	41.4

S. = strong; M. = medium; W. = weak.

$C_{19}H_{36}O_2$. Calculated. C 77.0, H 12.2, neutral equivalent 296
Found. " 77.5, " 12.2, " " 289

Amide—A sample of the pure acid was converted into the amide, which was recrystallized from 50 per cent aqueous ethanol; m.p. 79.2–81.2° (no depression on admixture with lactobacillamide).

$C_{19}H_{37}ON$. Calculated, C 77.2, H 12.6, N 4.7; found, C 77.5, H 13.0, N 4.7

Hydrazide—The hydrazide was prepared from the methyl ester and hydrazine hydrate in the usual manner. Following recrystallization from 50 per cent aqueous ethanol, the melting point was 59.6–61.2°. No depression of the melting point was observed on admixture with an authentic sample of the hydrazide of "phytomonic acid" (m.p. 56.4–57.6°) obtained from Dr. Velick.

DISCUSSION

Cells of *A. tumefaciens*, cultured on a medium containing sucrose and glutamic acid as the sole sources of carbon, contain 7.5 per cent of total lipides (based on the dry weight of the cells). This figure is in good agreement with the previously reported lipide content (6.7 per cent) of cells grown under comparable conditions (3). From the distillation curve (Fig. 1), it is apparent that the major fatty acid components contained 16, 18, and 19 carbon atoms. The C_{16} fatty acid was identified as palmitic acid; the occurrence of this acid in *A. tumefaciens* lipides has been reported previously (3). Saponification of the C_{18} ester fraction, which made up the bulk of the fatty acids, afforded essentially pure *cis*-vaccenic acid. Our unequivocal characterization of this acid, which is based on both physical and chemical criteria, fails to substantiate the previously reported presence of oleic acid in the lipides of *A. tumefaciens*.

Recently, we have studied the fatty acid spectrum of *L. arabinosus*, *L. casei*, and a *Streptococcus* species (7, 8, 11) and have identified *cis*-vaccenic acid as a major lipide constituent in all three. To date, *A. tumefaciens* lipides are the richest natural source of *cis*-vaccenic acid, which accounts for approximately 68 per cent of the total fatty acids of this organism. We were unable to detect stearic acid in the octadecanoic acid fraction, but the presence of trace amounts of this compound cannot be excluded.

Our findings point to a wide-spread distribution of *cis*-vaccenic acid in bacterial lipides and suggest a major difference between these lipides and those of higher animals and plants (which contain oleic acid as the major monoethenoid octadecanoic acid component). *cis*-Vaccenic acid is a minor constituent of horse brain lipides (12), and *trans*-vaccenic acid has been isolated from sheep and beef lipides and from butter fat (13, 14). Despite these observations, it has been generally assumed that the amount of *trans*-octadecenoic acids in animal fats is very small. By the use of infra-red spectroscopic techniques it was recently demonstrated (15) that beef lipides contain considerable quantities of *trans*-octadecenoic acids, mainly elaidic and *trans*-vaccenic acids. The origin of these trans isomerides is obscure at present, but it has been speculated that they may arise from the respective cis forms by oxidative cis-trans isomerization. According to this view, the formation of *trans*-vaccenic acid from oleic acid

involves, in addition to cis-trans isomerization, a selective shift of the 9,10 double bond to the 11,12 position. It is difficult to explain this highly specific migration of the double bond in terms of an oxidative mechanism. Our demonstration that certain bacteria are rich sources of *cis*-vaccenic acid points to intestinal bacteria as a likely source of the *trans*-vaccenic acid in the lipides of higher animals. The cis isomer elaborated by these organisms may undergo cis-trans isomerization during its absorption or transport to the tissues.

The fatty acid derived from the C_{19} ester fraction melted at 27.8–28.8° and was identical with lactobacillic acid from *L. arabinosus*. The identification was based on a comparison of the infra-red absorption spectra (Fig. 2), the x-ray diffraction patterns (Table I), and mixed melting point determinations on the acids and on two of their derivatives (amide and hydrazide). These findings clarify the chemical nature of "phytomonic acid."

In retrospect, it becomes apparent that Chargaff and Levine (1) were the first to recognize the presence of lactobacillic acid in nature. Anderson and his colleagues (2, 3) and Velick (4, 5) were in possession of impure samples, but failed to recognize the correct empirical composition and chemical nature of the acid. These workers resorted to catalytic hydrogenation for the separation of liquid-saturated fatty acids from liquid-unsaturated fatty acids, and their lactobacillic acid samples were impure. Contamination with 11- and 12-methyloctadecanoic acids (arising from the hydrogenolysis of the cyclopropane ring) seems to be responsible for the low melting points of these samples (7).

Pure samples of lactobacillic acid were isolated first from *L. arabinosus* and then from *L. casei* (7, 8) by a process which precluded the use of hydrogenation steps. Further studies (7, 9) of this pure material led to the establishment of its elementary composition and to the elucidation of its cyclopropane nature.

The presence of significant quantities of lactobacillic acid in the lipides of *A. tumefaciens*, an aerobe which differs markedly from the previously investigated lactobacilli, is of considerable interest and points to an important function of this cyclopropane fatty acid in bacterial metabolism.

SUMMARY

A reinvestigation of the chemical nature of the fatty acids of the plant pathogen *Agrobacterium tumefaciens* has led to results which differ significantly from those of previous workers. The lipides of this organism (7.5 per cent of dry weight of the cells) were found to contain palmitic acid (10 per cent), *cis*-vaccenic acid (68 per cent), lactobacillic acid (13 per cent), and unidentified high boiling materials (9 per cent). Presence of stearic acid could not be verified. The liquid-saturated fatty acid component

previously called "phytomonic acid" was shown to be lactobacillic acid. The importance of *cis*-vaccenic acid as major constituent of bacterial lipides is stressed, and the possible rôle of intestinal bacteria as a source of the vaccenic acid of animal fats is discussed.

The authors wish to express their appreciation to Dr. Brian Hutchings of the Lederle Laboratories Division of the American Cyanamid Company for the bacteria, to Dr. A. C. Hildebrandt of the Department of Plant Pathology of the University of Wisconsin for the original culture of *A. tumefaciens*, to Dr. E. S. Lutton of The Procter and Gamble Company for the x-ray diffraction patterns and their interpretations, and to Dr. S. F. Velick of the Department of Biochemistry, Washington University, St. Louis, for a sample of the hydrazide of "phytomonic acid."

BIBLIOGRAPHY

1. Chargaff, E., and Levine, M., *J. Biol. Chem.*, **124,** 195 (1938).
2. Geiger, W. B., Jr., and Anderson, R. J., *J. Biol. Chem.*, **129,** 519 (1939).
3. Velick, S. F., and Anderson, R. J., *J. Biol. Chem.*, **152,** 523 (1944).
4. Velick, S. F., *J. Biol. Chem.*, **152,** 533 (1944).
5. Velick, S. F., *J. Biol. Chem.*, **156,** 101 (1944).
6. Cavanna, R., and Ställberg-Stenhagen, S., *Chem. Abstr.*, **45,** 9470 (1951).
7. Hofmann, K., Lucas, R. A., and Sax, S. M., *J. Biol. Chem.*, **195,** 473 (1952).
8. Hofmann, K., and Sax, S. M., *J. Biol. Chem.*, **205,** 55 (1953).
9. Hofmann, K., Jucker, O., Miller, W. R., Young, A. C., Jr., and Tausig, F., *J. Am. Chem. Soc.*, **76,** 1799 (1954).
10. Swern, D., Billen, G. N., Findley, T. W., and Scanlan, J. T., *J. Am. Chem. Soc.*, **67,** 1786 (1945).
11. Hofmann, K., and Tausig, F., *J. Biol. Chem.*, **213,** 415 (1955).
12. Morton, I. D., and Todd, A. R., *Biochem. J.*, **47,** 327 (1950).
13. Bertram, S. H., *Biochem. Z.*, **197,** 433 (1928).
14. Nath, H., Barki, V. H., Elvehjem, C. A., and Hart, E. B., *J. Nutr.*, **36,** 761 (1948).
15. Swern, D., Knight, H. B., and Eddy, C. R., *J. Am. Oil Chem. Soc.*, **29,** 44 (1952).

THE FATE OF INGESTED ACETYL-L- AND D-TRYPTOPHAN IN THE HUMAN SUBJECT

By RALPH R. LANGNER and CAROL M. VOLKMANN

(From the Department of Biochemistry, The University of Texas Dental Branch, Houston, Texas)

(Received for publication, July 2, 1954)

The metabolism of acetyltryptophan has been studied by various workers. Baldwin and Berg (1) and Rose (2) have reported that acetyl-D-tryptophan will not maintain nitrogen balance in the adult human; however, the acetylated L isomer was equal to an equivalent amount of L-tryptophan in this respect. In contrast with these results, Albanese *et al.* (3) report that, with the exception of a 5 per cent urinary loss, all the orally administered acetyl-DL-tryptophan was available to man and that acetyl-D-tryptophan, when given as the racemic mixture, will support a positive nitrogen balance in human infants (4). They (3, 4) cite, for evidence of acetyl-D-tryptophan utilization, that there was (*a*) no indigo red production upon the addition of 0.1 N iodine to urine excreted during the succeeding 6 hours, and that (*b*) acetyl-L-tryptophan caused higher excretion of tryptophan than did acetyl-DL-tryptophan. They conclude that "metabolic deacetylation of acetyl-*d*-tryptophane results in the formation of an indole derivative which, unlike that formed in the metabolism of *d*-tryptophane, is available to man." Luck *et al.* (5) found that 70 to 83 per cent of intravenously injected acetyl-DL-tryptophan was excreted in the urine within 6 hours. As Albanese *et al.* (4) point out, the method of administration used by the latter group (5) may overwhelm the enzyme systems responsible for the utilization of the acetyl derivatives.

With the above information at hand, we decided to administer separately the D and L isomers of acetyltryptophan to adult humans in order to gain more information as to the fate of the derivatives. Our data show that approximately 75 per cent of the ingested acetyl-D-tryptophan was not absorbed and was excreted in the feces.

Methods and Results

Acetyl-DL-tryptophan was resolved by using the brucine salt as directed by Shabica and Tishler (6). The $[\alpha]_D^{25}$ was $-29.0°$ for the D isomer and $+29.2°$ for the L isomer (1 per cent in H_2O plus 1 equivalent of NaOH) (6–8). The method of administration was similar to that employed by Albanese *et al.* (4), 0.01 mole (2.46 gm.) of the acetyl derivatives of D- or L-tryptophan having been ingested. We found that suspension of the

crystals in a milk shake made the ingestion more pleasant. The urine was collected for 24 hours and analyzed colorimetrically for acetyltryptophan or free tryptophan by using the Bates *p*-dimethylaminobenzaldehyde procedure (9). Both free tryptophan and acetyltryptophan respond to the Bates method. Acetyltryptophan was used for the standard solution and all samples were read in the Klett-Summerson photoelectric colorimeter with the No. 54 filter. The values were calculated by using a standard which gave readings in the same range as the unknown. All tests were repeated within 24 hours after the initial determination. Normal urine values for tryptophan were from 29 to 44 mg., which are in agreement with those reported elsewhere (10–13).

After the ingestion of 2.46 gm. of acetyl-D-tryptophan, only 0.520 gm. was excreted in the urine. To elucidate what was happening, the urine was chromatographed on Whatman No. 4 paper by the solvent system of Mason and Berg (14): methanol, butanol, benzene, and water in a 2:1:1:1 ratio. The only spot which differed from normal had an R_F value of 0.73, which corresponded with that of acetyltryptophan. When sprayed with Ehrlich's *p*-dimethylaminobenzaldehyde solution and warmed, the spot turned purple, as do indole derivatives. No spot was detected for D-tryptophan, as described by Langner and Berg (15), after the ingestion of D-tryptophan; therefore, it is assumed that the urine did not contain the deacetylated amino acid.

On the basis of the above results, it occurred to us that perhaps the acetyl-D-tryptophan was not absorbed through the intestinal wall and was being excreted in the feces. Again 2.46 gm. of acetyl-D-tryptophan were administered and the urine was collected. Carmine was also ingested to mark the feces. The feces were extracted three times with 250 ml. of hot alcohol, then further diluted to 1000 ml. Although the extract was brown in color, a control tube containing everything except the *p*-dimethylaminobenzaldehyde reagent served as a satisfactory blank. No detectable amount of acetyltryptophan (or tryptophan) was found in the feces normally; therefore, the stools were analyzed until a blank reading was obtained. This occurred within 72 hours. In order to check the extraction procedure, 1 gm. of acetyltryptophan was mixed with a normal stool; 95 $\pm$ 2 per cent could be accounted for by using this method. Our data indicate that 75 per cent of the acetylated D isomer was not absorbed, whereas all but a trace of the acetyl-L-tryptophan passed through the intestinal wall. Table I gives typical results of different test periods. In examining the L isomer (Table I), the same procedures were used.

Paper chromatograms of both the urine and feces extracts showed only acetyltryptophan. There was no evidence of deacetylation of either isomer, although acetyl-L-tryptophan is presumed to be deacetylated before

it is utilized. The small amount of acetyl-L-tryptophan found in the urine was most probably excreted before deacetylation occurred.

The presence of a small amount of acetyl-L-tryptophan in the feces suggested the incubation of a normal stool with acetyl-L-tryptophan in order to ascertain whether intestinal bacteria were destroying the acetyl-L-tryptophan. The stool was stirred with 100 ml. of water and 1 gm. of acetyl-L-tryptophan and incubated at 37° for 48 hours. When this was

TABLE I

Excretion of Acetyl-D- and Acetyl-L-tryptophan in Urine and Feces after Ingestion of 0.01 Mole (2.46 Gm.) of Acetylated Amino Acids (Corrected for Normal Excretion)

Isomer	Subject	Time after ingestion of amino acids	Urine*	Feces
		hrs.	*mg.*	*mg.*
Acetyl-D-tryptophan	L.	24	670	1210
		48	0	552
		72	0	0
	L.	24	660	1230
		48	0	580
		72	0	0
	S.	24	373	1665
		48	0	146
		72	0	0
	O.	24	211	514†
		48	0	635
		72	0	105
Acetyl-L-tryptophan	L.	24	0	100
		48	0	0
	L.	24	100	130
		48	0	36

* All of the acetyltryptophan was excreted within 20 hours.

† Very hard stool, and the extraction was probably not complete.

extracted with alcohol, all of the added acetyltryptophan was measured colorimetrically. When acid extracts (0.1 N HCl) of the incubated stool were chromatographed for free tryptophan, no spot was detected. When media containing only acetyl-L- or acetyl-D-tryptophan as the nitrogen source were seeded with a stool specimen, no growth resulted.

Birnbaum *et al.* (16) have used, for resolution, acylase preparations from hog kidney which deacetylated both acetyl and chloroacetyl derivatives of many L-amino acids. The acetylated D isomers were unaffected. The reaction of the crude hog kidney enzyme with acetyl-L-tryptophan was negligible (17), but was increased ten times when a purified enzyme was used (16). By using paper chromatography, we have studied the presence

crystals in a milk shake made the ingestion more pleasant. The urine was collected for 24 hours and analyzed colorimetrically for acetyltryptophan or free tryptophan by using the Bates *p*-dimethylaminobenzaldehyde procedure (9). Both free tryptophan and acetyltryptophan respond to the Bates method. Acetyltryptophan was used for the standard solution and all samples were read in the Klett-Summerson photoelectric colorimeter with the No. 54 filter. The values were calculated by using a standard which gave readings in the same range as the unknown. All tests were repeated within 24 hours after the initial determination. Normal urine values for tryptophan were from 29 to 44 mg., which are in agreement with those reported elsewhere (10–13).

After the ingestion of 2.46 gm. of acetyl-D-tryptophan, only 0.520 gm. was excreted in the urine. To elucidate what was happening, the urine was chromatographed on Whatman No. 4 paper by the solvent system of Mason and Berg (14): methanol, butanol, benzene, and water in a 2:1:1:1 ratio. The only spot which differed from normal had an R_F value of 0.73, which corresponded with that of acetyltryptophan. When sprayed with Ehrlich's *p*-dimethylaminobenzaldehyde solution and warmed, the spot turned purple, as do indole derivatives. No spot was detected for D-tryptophan, as described by Langner and Berg (15), after the ingestion of D-tryptophan; therefore, it is assumed that the urine did not contain the deacetylated amino acid.

On the basis of the above results, it occurred to us that perhaps the acetyl-D-tryptophan was not absorbed through the intestinal wall and was being excreted in the feces. Again 2.46 gm. of acetyl-D-tryptophan were administered and the urine was collected. Carmine was also ingested to mark the feces. The feces were extracted three times with 250 ml. of hot alcohol, then further diluted to 1000 ml. Although the extract was brown in color, a control tube containing everything except the *p*-dimethylaminobenzaldehyde reagent served as a satisfactory blank. No detectable amount of acetyltryptophan (or tryptophan) was found in the feces normally; therefore, the stools were analyzed until a blank reading was obtained. This occurred within 72 hours. In order to check the extraction procedure, 1 gm. of acetyltryptophan was mixed with a normal stool; 95 $\pm$ 2 per cent could be accounted for by using this method. Our data indicate that 75 per cent of the acetylated D isomer was not absorbed, whereas all but a trace of the acetyl-L-tryptophan passed through the intestinal wall. Table I gives typical results of different test periods. In examining the L isomer (Table I), the same procedures were used.

Paper chromatograms of both the urine and feces extracts showed only acetyltryptophan. There was no evidence of deacetylation of either isomer, although acetyl-L-tryptophan is presumed to be deacetylated before

it is utilized. The small amount of acetyl-L-tryptophan found in the urine was most probably excreted before deacetylation occurred.

The presence of a small amount of acetyl-L-tryptophan in the feces suggested the incubation of a normal stool with acetyl-L-tryptophan in order to ascertain whether intestinal bacteria were destroying the acetyl-L-tryptophan. The stool was stirred with 100 ml. of water and 1 gm. of acetyl-L-tryptophan and incubated at 37° for 48 hours. When this was

TABLE I

Excretion of Acetyl-D- and Acetyl-L-tryptophan in Urine and Feces after Ingestion of 0.01 Mole (2.46 Gm.) of Acetylated Amino Acids (Corrected for Normal Excretion)

Isomer	Subject	Time after ingestion of amino acids	Urine*	Feces
		hrs.	*mg.*	*mg.*
Acetyl-D-tryptophan	L.	24	670	1210
		48	0	552
		72	0	0
	L.	24	660	1230
		48	0	580
		72	0	0
	S.	24	373	1665
		48	0	146
		72	0	0
	O.	24	211	514†
		48	0	635
		72	0	105
Acetyl-L-tryptophan	L.	24	0	100
		48	0	0
	L.	24	100	130
		48	0	36

* All of the acetyltryptophan was excreted within 20 hours.

† Very hard stool, and the extraction was probably not complete.

extracted with alcohol, all of the added acetyltryptophan was measured colorimetrically. When acid extracts (0.1 N HCl) of the incubated stool were chromatographed for free tryptophan, no spot was detected. When media containing only acetyl-L- or acetyl-D-tryptophan as the nitrogen source were seeded with a stool specimen, no growth resulted.

Birnbaum *et al.* (16) have used, for resolution, acylase preparations from hog kidney which deacetylated both acetyl and chloroacetyl derivatives of many L-amino acids. The acetylated D isomers were unaffected. The reaction of the crude hog kidney enzyme with acetyl-L-tryptophan was negligible (17), but was increased ten times when a purified enzyme was used (16). By using paper chromatography, we have studied the presence

of acylases in liver and kidney of various animal species and have found a measurable amount of deacetylating enzyme specific for the L isomer of acetyltryptophan in liver and kidney of the rabbit, hog, cow, rat, mouse, and hamster. The tissue (1 gm.) was homogenized in 2 ml. of Ringer's solution by using a glass homogenizer. The resulting solution (1 ml.) was incubated overnight with 0.005 mole of acetyl-L- or acetyl-D-tryptophan and phosphate buffer at pH 7.4. The filtrate was spotted on Whatman No. 4 paper and chromatographed, the technique described earlier being used. The deacetylation was determined by the disappearance of the acetyltryptophan spot or the appearance of tryptophan. No deacetylation of the D isomer was observed in any case.

DISCUSSION

The data presented here indicate a reason why Albanese *et al.* and Luck *et al.* differed in their findings. The results also give evidence that acetyl-D-tryptophan is not utilized by man, a conclusion at variance with that of Albanese *et al.* (3, 4). Although the small amount of tryptophan required to maintain nitrogen balance could be overlooked by experimental error, this possibility does not seem justified in the light of earlier findings with acetyl-D-tryptophan or D-tryptophan (1, 2). The evidence regarding the production of indigo red cited by Albanese *et al.* (3) has been incorrectly interpreted, since acetyltryptophan does not respond with iodine to produce indigo red (18, 19). The fact that an ingested test compound (or its metabolites) is not found in the urine does not necessarily mean that the compound is utilized. It may be important to use the resolved isomers if correct conclusions are to be drawn. The influence of the L isomer on the utilization of the D enantiomorph of tryptophan and histidine in growth studies with mice has been reported (20).

The present results raise the question as to what happens to the ingested acetyl-L-tryptophan. There are three possibilities: (*a*) It is deacetylated when passing through the gastrointestinal tract and is absorbed as free L-tryptophan, (*b*) it is absorbed as acetyl-L-tryptophan and then deacetylated, and (*c*) both may occur. Since paper chromatography and colorimetric analyses show a smaller amount of acetyl-L-tryptophan than of acetyl-D-tryptophan in the urine, the first possibility seems improbable but should not be overlooked. Both isomers have the same physical properties with the exception of optical rotation. If the first enzyme responsible for the preferential utilization of acetyl-L-tryptophan were in the liver or some other organ, more of the acetyl-D-tryptophan would be absorbed and excreted in the urine. Since the major portion of the acetylated D isomer was found in the feces, the enzymes responsible for the absorption and the consequent utilization of acetyl-L-tryptophan must

attack the molecule before or during absorption. From the evidence cited for the difference in the absorption of the two isomers of acetyltryptophan, we postulate an enzymatic transfer of acetyl-L-tryptophan across the intestinal mucosa, rather than simple diffusion. Further studies are in progress to gain more knowledge concerning the absorption mechanism of acetyltryptophan and other amino acids.

SUMMARY

Acetyltryptophan was resolved into its two isomers. Each isomer was ingested separately, and the amounts excreted in the urine and in the feces were determined. No evidence was found for the metabolism of acetyl-D-tryptophan, and approximately 75 per cent was excreted in the feces. Only 5 to 10 per cent of acetyl-L-tryptophan could be accounted for in the feces. An enzymatic absorption of acetyl-L-tryptophan has been suggested from this evidence. Differences in earlier reports as to the fate of acetyl-D-tryptophan have been discussed.

BIBLIOGRAPHY

1. Baldwin, H. R., and Berg, C. P., *J. Nutr.*, **39,** 203 (1949).
2. Rose, W. C., *Federation Proc.*, **8,** 546 (1949).
3. Albanese, A. A., Frankston, J. E., and Irby, V., *J. Biol. Chem.*, **160,** 31 (1945).
4. Albanese, A. A., Davis, V. I., and Lein, M., *J. Biol. Chem.*, **172,** 39 (1948).
5. Luck, J. M., Boyer, P. D., and Hall, V. E., *Proc. Soc. Exp. Biol. and Med.*, **62,** 177 (1946).
6. Shabica, A. C., and Tishler, M., *J. Am. Chem. Soc.*, **71,** 3251 (1949).
7. du Vigneaud, V., and Sealock, R. R., *J. Biol. Chem.*, **96,** 511 (1932).
8. Berg, C. P., *J. Biol. Chem.*, **100,** 79 (1933).
9. Bates, R. W., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **119,** p. vii (1937).
10. Berg, C. P., and Rohse, W. G., *J. Biol. Chem.*, **170,** 725 (1947).
11. Schweigert, B. S., Sauberlich, H. E., Elvehjem, C. A., and Baumann, C. A., *J. Biol. Chem.*, **164,** 213 (1946).
12. Frankl, W., and Dunn, M. S., *Arch. Biochem.*, **13,** 93 (1947).
13. Schweigert, B. S., Sauberlich, H. E., and Elvehjem, C. A., *Science*, **102,** 275 (1945).
14. Mason, M., and Berg, C. P., *J. Biol. Chem.*, **188,** 783 (1951).
15. Langner, R. R., and Berg, C. P., *Federation Proc.*, **13,** 247 (1954).
16. Birnbaum, S. M., Levintow, L., Kingsley, R. B., and Greenstein, J. P., *J. Biol. Chem.*, **194,** 455 (1952).
17. Fodor, P. J., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **178,** 503 (1949).
18. Fearon, W. R., and Boggust, W. A., *Biochem. J.*, **46,** 62 (1950).
19. Langner, R. R., and Berg, C. P., *J. Biol. Chem.*, in press.
20. Celander, D. R., and Berg, C. P., *J. Biol. Chem.*, **202,** 339 (1953).

ENZYMATIC SYSTEM SYNTHESIZING SULFURIC ACID ESTERS OF PHENOLS*

By R. H. De Meio, Martha Wizerkaniuk, and I. Schreibman

(From the Department of Biochemistry, Jefferson Medical College, Philadelphia, Pennsylvania)

(Received for publication, August 11, 1954)

Liver preparations have been shown (2–6) to catalyze the synthesis of the sulfuric acid esters of phenols. The enzyme system is present in the soluble portion of the liver preparations and requires only adenosinetriphosphate (ATP), Mg^{++}, and inorganic sulfate (4, 5).

Bernstein and McGilvery (6) demonstrated that the formation of the sulfuric acid ester is preceded by activation of the sulfate and have indicated the possibility that two enzymes might be involved. We have been able to confirm their results on the formation of the "active sulfate" and to separate two activities from ammonium sulfate fractions of the soluble rat liver preparations (1). One of the activities, designated the "activating enzyme," remains after heating the preparation at 52°. The other ("transferring enzyme") remains after dialysis against or contact with 0.03 M glycylglycine.

EXPERIMENTAL

The tissue preparation was obtained from liver of male albino rats (Albino Farms, Red Bank, New Jersey) killed by decapitation. A 20 per cent liver homogenate in 0.1 M potassium phosphate buffer, pH 7, was prepared with a Potter-Elvehjem tissue homogenizer. The "microsome-free" solution was obtained from this homogenate as described in a previous paper (4). The solution was lyophilized and kept at −20°. The activity of this preparation has not changed appreciably for a period of a year. For further use the lyophilized material was brought to the initial concentration with distilled water. The medium used for incubation had the following composition: 0.05 M potassium phosphate buffer, pH 7, 0.005 M Mg^{++}, 0.015 M $SO_4^{=}$, 0.005 M ATP,[1] and 0.24×10^{-3} M *p*-nitrophenol. *p*-Nitrophenol and protein were determined by methods described elsewhere (3, 4).

* These studies were aided in part by contract Nonr-229(OO) between the Office of Naval Research, Department of the Navy, and Jefferson Medical College, and by research grant G-3839 from the National Institutes of Health, United States Public Health Service. Presented in part at the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April 12–16, 1954 (1).

[1] Disodium ATP from the Pabst Laboratories, Milwaukee, Wisconsin, was used in all the experiments.

XUM

Ammonium Sulfate Fractionation—The liver "microsome-free" solution was fractionated with neutralized ammonium sulfate in the cold (between 0° and 5°). The fraction that precipitated between 1.7 and 2.3 M ammonium sulfate (1.7 to 2.3 M) was the most active, in agreement with the findings of Bernstein and McGilvery (5). The fraction precipitating between 1.2 and 1.7 M ammonium sulfate showed lower activity. A third fraction (2.3 to 3 M) was inactive by itself, but it increased the activity of the 1.2 to 1.7 M fraction. The factor responsible for this behavior of the 2.3 to 3 M fraction is thermolabile. We believe this to be the "transferring enzyme" because of its properties.

Fractions of slightly greater specific activity were obtained from acetone powder prepared from the "microsome-free" solution.

Demonstration of Two Activities—If the active ammonium sulfate fractions (1.2 to 1.7 M and 1.7 to 2.3 M) were incubated at 37.5° for 30 minutes in the presence of 10 to 15 per cent alcohol or were heated at 52° for 15 minutes, the activity was lost (1). Dialysis of the ammonium sulfate fraction against 0.03 M glycylglycine, pH 7.4, for 24 hours at 0–5° also resulted in an inactive preparation. Activity was restored to a large extent when the heat- and glycylglycine-inactivated preparations were combined (Table I).

Inactivation by glycylglycine was also obtained if the dialyzed ammonium sulfate fraction was made 0.03 M in glycylglycine and left in contact for 24 hours at 0–5°. Prolonged dialysis against 0.02 M phosphate buffer, pH 7.4, did not restore the activity. The presence of 0.075 M phosphate, pH 7.4, prevented the inactivation by glycylglycine, and with 0.023 M sulfate 75 per cent of the activity was still present at the end of contact. Glycylglycylglycine produced the same effect as glycylglycine, benzoylglycylglycine was not effective, and glycylglycine ethyl ester produced 20 per cent inactivation. The following compounds were effective in concentrations ranging from 2.5 to 7.5×10^{-2} M and in decreasing order of activities (from 77 to 13 per cent inactivation): putrescine, tris(hydroxymethyl)-aminomethane, cadaverine, L-cysteine, alanylglycine, glycine, L-threonine, L-lysine, L-alanine, L-histidine, DL-ornithine, L-arginine, and glutathione. Addition of pyridoxal phosphate had no effect on the inactivation. 0.001 M sodium ethylenediaminetetraacetate[2] and 0.001 to 0.03 M *N*,*N*-dihydroxyethylglycine[2] did not inactivate the enzyme preparation.

Activating and Transferring Enzymes—As indicated by the observations of Bernstein and McGilvery (6), the formation of the sulfuric ester of phenols is preceded by a step of activation. Their results suggest that when the enzyme preparation is incubated with sulfate, ATP, and Mg^{++} in the absence of phenol, there is accumulation of an intermediate "active

[2] Obtained from the Bersworth Chemical Company, Framingham, Massachusetts.

sulfate." This is indicated by the fact that, on addition of phenol to the previously incubated samples, the initial rate of formation of phenyl sulfate

TABLE I

Inactivation by Heat and Glycylglycine Treatment of System That Catalyzes Synthesis of p-Nitrophenyl Sulfate

1.2 to 2.3 M ammonium sulfate fraction dialyzed for 17 hours against neutralized triple distilled water (dialyzed fraction) and left in contact with 0.03 M glycylglycine, pH 7.4, for 24 hours at 0–5° (glycylglycine-treated). Ammonium sulfate fraction heated for 15 minutes at 52° (heat-treated). Activity tested according to the method described in the text.

Preparation	Activity*
Dialyzed fraction	53.4
Glycylglycine-treated fraction	0
Heat-treated fraction	1.6
Glycylglycine-treated + heat-treated	46.4

* Micrograms of *p*-nitrophenol bound in 1 hour.

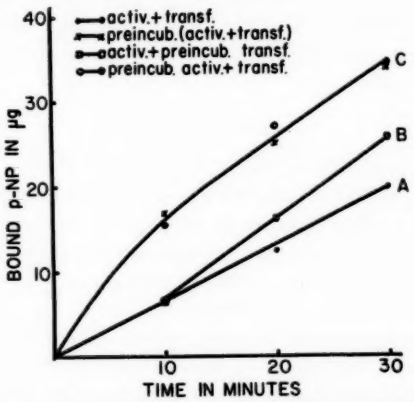


FIG. 1. Activating and transferring enzymes in the synthesis of *p*-nitrophenyl sulfate. Activating and transferring enzymes obtained from 1.2 to 2.3 M ammonium sulfate fraction, followed by heating 15 minutes at 52° or by 24 hours contact with 0.03 M glycylglycine at 0–5°, respectively. Medium, 0.05 M potassium phosphate buffer, pH 7, 0.005 M Mg^{++}, 0.015 M K_2SO_4, 0.005 M ATP, *p*-nitrophenol 0.72 µmole. Preincubation without phenol for ½ hour; incubation for 1 hour. Temperature 37.5°; 3 ml. per vessel.

is more rapid than without preincubation. A similar technique has been used by us.

The preparation to be tested was incubated for half an hour in the regu-

lar medium with sulfate, ATP, and Mg^{++}, but without phenol (preincubated preparation). A greater rate of formation of *p*-nitrophenyl sulfate on incubation of this preparation with *p*-nitrophenol would indicate accumulation of an "active sulfate" intermediate and the presence of the enzyme required for activation (activating enzyme). Some representative results are presented in Fig. 1. Little or no increase in rate is observed when the preparation obtained by inactivation with glycylglycine is preincubated and then incubated with the preparation submitted to the heat treatment by adding this together with the phenol (compare Curves A and B). This indicates that the glycylglycine-treated preparation does not contain active activating enzyme. On the other hand, preincubation of the heat-treated preparation with sulfate, ATP, and Mg^{++} leads to an increase in rate on addition of glycylglycine-treated enzyme (Curve C) compared with a similar sample without preincubation (Curve A). This suggests that the heated preparation contains the activating enzyme.

We have found that the "active sulfate" intermediate is stable on 20 hours of dialysis against 0.1 M potassium phosphate buffer, pH 7.4. We have been unable to detect formation of glucuronides by the ammonium sulfate fractions active in the formation of sulfates. Hydroxylamine, hydrazine, and semicarbazide (0.03 to 0.01 M) had no effect on the synthesis of *p*-nitrophenyl sulfate.

DISCUSSION

The preliminary results of Bernstein and McGilvery (6) indicated that at least two steps were required for the synthesis of phenyl sulfates. The first step involves the formation of an "active sulfate" intermediate and the second the condensation of the intermediate with the phenol. They reached their conclusion on the basis of kinetic data. We have been able to separate the two activities. The first, due to the activating enzyme, remains after heating for 15 minutes at 52°, a treatment that inactivates the other enzyme. We prefer to think of the second step as a transfer process. The sulfate of the active intermediate seems to be quite firmly attached to the enzyme, as it is not freed on prolonged dialysis. It appears as though the sulfate were transferred from the intermediate to the substrate. The activity of the enzyme involved in this process can be demonstrated in preparations kept in contact for 24 hours with 0.03 M glycylglycine. This treatment inactivates preferentially the activating enzyme. The fact that sulfate and phosphate protect the preparation against the action of glycylglycine might be due to a binding of these ions to the active center or centers. The action of glycylglycine is apparently not the result of a chelating effect because sodium ethylenediaminetetraacetate and *N*,*N*-dihydroxyethylglycine have no influence on the activity of the preparation.

SUMMARY

The presence of two enzymes involved in the synthesis of the sulfuric acid esters of phenols has been demonstrated: (*a*) an activating enzyme, responsible for the formation of an active sulfate intermediate, and (*b*) a transferring enzyme, involved in the transfer of sulfate from the intermediate to the substrate. The active sulfate intermediate is not dialyzable. These preparations do not form the glucuronide. The effect of other inhibitors is presented and discussed.

We should like to express our gratitude to Merck and Company, Inc., Rahway, New Jersey, for supplying us with pyridoxal phosphate.

BIBLIOGRAPHY

1. De Meio, R. H., Wizerkaniuk, M., and Schreibman, I., *Federation Proc.*, **13,** 198 (1954).
2. De Meio, R. H., and Tkacz, L., *J. Biol. Chem.*, **195,** 175 (1952).
3. De Meio, R. H., *Acta physiol. Latinoamer.*, **2,** 195 (1952).
4. De Meio, R. H., Wizerkaniuk, M., and Fabiani, E., *J. Biol. Chem.*, **203,** 257 (1953).
5. Bernstein, S., and McGilvery, R. W., *J. Biol. Chem.*, **198,** 195 (1952).
6. Bernstein, S., and McGilvery, R. W., *J. Biol. Chem.*, **199,** 745 (1952).

FORMALDEHYDE DEHYDROGENASE, A GLUTATHIONE-DEPENDENT ENZYME SYSTEM*

By PHILIPP STRITTMATTER† and ERIC G. BALL

(From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts)

(Received for publication, August 16, 1954)

It has been generally assumed that formaldehyde oxidation by animal tissue is catalyzed by one of the enzymes that is known to catalyze the oxidation of aldehyde groups (2–5), and most of the studies on aldehyde oxidation have been concerned primarily with enzymes that attack aldehydes other than formaldehyde. Data on the activity of these systems towards formaldehyde often were incidental information acquired in determining substrate specificities. Furthermore, few attempts seem to have been made to determine the conditions for optimal oxidation of formaldehyde *per se*. For these reasons, and in view of the involvement of 1-carbon compounds in various synthetic processes, the oxidation of formaldehyde by animal tissue preparations has been reinvestigated.

The experiments to be described here show that formaldehyde is oxidized by a DPN[1]-dependent dehydrogenase distinct from that which is active for acetaldehyde, and that GSH is a specific cofactor in this formaldehyde dehydrogenase system. A method for the partial, functional purification of this enzyme is given and some of the properties of this dehydrogenase system are reported.

Methods

Dehydrogenase activity for ethanol, acetaldehyde, and formaldehyde was measured by following the rate of reduction of DPN to $DPN_{red.}$ at 340 mμ, with a Beckman model DU spectrophotometer.

* This work was supported in part by funds received from the Eugene Higgins Trust through Harvard University. There is a preliminary report of this work (1) and it also appears in more detail in the thesis submitted by P. A. J. Strittmatter to Harvard University, December 1, 1953, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

† Predoctoral Fellow of the Atomic Energy Commission, 1951–53. Present address, Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri.

[1] The following abbreviations have been used: DPN, diphosphopyridine nucleotide; $DPN_{red.}$, reduced DPN; TPN, triphosphopyridine nucleotide; GSH, reduced glutathione; CoA, coenzyme A; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid.

XUM

Zinc glycinate was prepared as follows:[2] An aqueous suspension of 1 M ZnO and 3 M glycine was heated to 85–95° and stirred until almost all of the ZnO had dissolved. This solution was then quickly filtered while hot and the filtrate allowed to cool to 5°. The zinc glycinate crystals which formed were collected on a Büchner funnel, washed with cold water, and dried at 90° overnight. This dried zinc glycinate has an empirical formula of 1 Zn, 2 glycine, and 1 molecule of water, and a molecular weight of 233. This preparation was used as a 0.3 M solution in 0.3 M glycine.

The calcium phosphate gel was prepared by mixing 300 cc. of 1 M calcium acetate with 450 cc. of 1 M disodium phosphate. The gel was centrifuged at 1500 × *g* for 15 minutes and the supernatant fluid decanted and discarded. The gel was washed with 2 volumes of distilled water and centrifuged, and the supernatant fluid was discarded. The volume of gel was about 350 cc. and the pH approximately 6.6 to 7.0.

A standard solution of formaldehyde was prepared by the method of Weinberger (6) and its concentration, determined by the chromotropic acid method, was 3.33 M. For most experiments a dilute solution containing 3.33 or 6.66 μmoles per cc. was prepared daily from this stock solution.

Formate was determined by distilling 1 cc. of reaction mixture plus 1 drop of 12 N HCl to dryness in a micro still (7) and analyzing an aliquot of the distillate by the micromethod of Grant (8). Formaldehyde was determined by the chromotropic acid procedure adapted from the last step of Grant's formate method.

Protein content of enzyme preparations was determined by measuring the absorption at 280 and 260 mμ, correcting for nucleic acid as described by Warburg and Christian (9), and also by precipitating the protein with 12 per cent TCA, washing the precipitate with 70 per cent ethanol, and drying at 100° to constant weight.

Preparation of Beef Liver Formaldehyde Dehydrogenase—Either fresh beef liver or tissue stored as long as 1 month at −20° has been used equally satisfactorily. All steps were carried out at 5–10° unless otherwise indicated.

Step 1—300 gm. of beef liver, cut into small pieces, were homogenized in a Waring blendor in 75 gm. portions for 2 minutes with a total volume of 900 cc. of distilled water. Slowly with stirring, 200 cc. of 0.3 M zinc glycinate in 0.3 M glycine (this solution was at room temperature) were added over a period of 5 minutes. The pH of the suspension was then adjusted to 6.4 to 6.5 by the dropwise addition, with vigorous stirring, of approximately 50 to 55 cc. of 1 N HCl. The first 20 cc. of HCl were added in about 5 minutes to bring the pH rapidly to 7.4; the remainder was added very slowly over a period of about 40 minutes. The pH measurements

[2] Private communication from F. R. N. Gurd and P. E. Wilcox.

were made by diluting an aliquot of the suspension with 5 volumes of distilled water and reading the apparent pH with a Beckman pH meter. The suspension was then stirred slowly for 3 hours and finally centrifuged at about 1500 × *g* for 45 minutes or until the precipitate was well packed. The volume of the slightly red, opalescent supernatant fluid containing the enzyme, was about 700 to 800 cc.

Step 2—The supernatant fluid from Step 1 was mixed with about $\frac{1}{3}$ volume of calcium phosphate gel. The pH was then adjusted quickly to pH 7.1 to 7.2 with about 3 to 4 cc. of 2 N NaOH added dropwise. After being stirred slowly for 30 minutes, this suspension was centrifuged at 1500 × *g* for 15 minutes, yielding about 700 to 800 cc. of a light yellow opalescent supernatant fluid. The supernatant fluid was saved and the gel discarded.

Step 3—To the enzyme solution from Step 2, 0.475 volume of acetone was added slowly with stirring. At all times, the temperature was kept as near the freezing point as possible (0 to −20°). Acetone was prechilled to −40 to −60° in a dry ice bath and added very slowly to the enzyme preparation which was cooled in a dry ice bath to a point just above freezing. The resulting precipitate was centifuged at 1500 × *g* for 15 minutes at −5° and discarded. The temperature of preparations during centrifugation in a −5° cold room should not be allowed to rise above −3°. To the supernatant fluid cooled to −10°, another 0.72 volume of acetone (based on the volume of enzyme solution from Step 2) was added slowly with cooling. The final temperature of this mixture was −15 to −20°. The precipitate, centrifuged at 1500 × *g* for 15 minutes at −5°, was transferred to a 500 cc. round bottomed flask with about 50 cc. of ice-cold glass-distilled water and immediately lyophilized. The yield of very light cream-colored powder was about 3 to 4 gm. This enzyme preparation (acetone precipitate) could be stored as the dry powder for months at −20° without loss of activity. For activity assays, 50 mg. of powder were extracted with 5 cc. of glass-distilled water at 5° for 1 hour. The slight sediment of insoluble material obtained on centrifuging was discarded and aliquots of the clear enzyme solution (usually 0.3 cc.) were used in assay systems.

Results

Preliminary Experiments—It can readily be shown that the oxidation of either formaldehyde or acetaldehyde in crude liver extracts is dependent on the presence of DPN. The method of choice for following these reactions is the measurement of the rate of DPN reduction spectrophotometrically. However, the presence of alcohol dehydrogenase in such crude tissue preparations results in a coupled oxidation and reduction of acetaldehyde (5), and under these conditions the rate of DPN reduction is not a

true measure of the rate of aldehyde oxidation. In preliminary experiments, therefore, the Thunberg method (10) was used to measure the rate of methylene blue reduction by the two aldehydes. These studies showed that the addition of GSH accelerated the oxidation of formaldehyde but not of acetaldehyde by chicken, beef, rat, or pigeon liver extracts and by rat or pigeon kidney extracts.

The Thunberg method was abandoned in favor of the spectrophotometric procedure when it was found that zinc glycinate treatment permitted the removal of alcohol dehydrogenase activity from tissue preparations, thus eliminating the coupled oxidation and reduction of acetaldehyde. It is

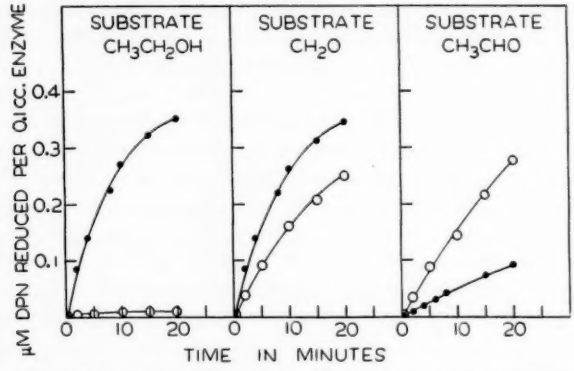


FIG. 1. Spectrophotometric assays of zinc glycinate preparation. System, aerobic, temperature 23–26°, 0.1 cc. of enzyme, 6 μmoles of GSH, 0.8 μmole of DPN, 1 mg. of substrate, 0.1 M sodium pyrophosphate to 3.0 cc. (pH 9.2). ○, zinc glycinate preparation; ●, crude liver extract. Chicken liver was the enzyme source.

for this reason that the zinc glycinate treatment constitutes Step 1 of the enzyme preparation method. Fig. 1 summarizes the effect of the zinc glycinate treatment on the ability of a chicken liver preparation to catalyze the oxidation of ethanol, formaldehyde, and acetaldehyde. The ethanol dehydrogenase has been almost completely eliminated by zinc glycinate precipitation. As a consequence, the coupled reaction involving acetaldehyde was essentially eliminated and the rate of DPN reduction with acetaldehyde was increased 3-fold. This rate now presumably represents more nearly the true activity of the acetaldehyde dehydrogenase system. In the case of formaldehyde oxidation, on the other hand, this treatment resulted in a decrease of about 25 per cent in the rate of DPN reduction. This fact suggests that formaldehyde does not as readily undergo a coupled reaction, involving reduction of formaldehyde to methanol in the presence of alcohol dehydrogenase, analogous to acetaldehyde reduction to ethanol.

With zinc glycinate preparations, it was possible to obtain evidence that two distinct enzyme systems are involved in the oxidation of acetaldehyde and formaldehyde. It can be seen in Fig. 2 that the rate of DPN reduction in a tube containing both formaldehyde and acetaldehyde (Curve 3) was approximately equal to the sum of the rates of DPN reduction observed in systems in which formaldehyde and acetaldehyde were assayed separately at optimal substrate concentrations (solid line). This is as would be expected for a solution containing two aldehyde enzymes.

A separation of the acetaldehyde and formaldehyde dehydrogenases can be achieved by the preferential adsorption of the acetaldehyde enzyme on

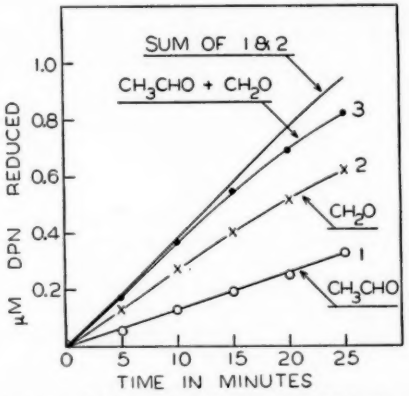


FIG. 2. Additive rates of formaldehyde and acetaldehyde oxidation. System, aerobic, temperature 23–26°, 0.3 cc. of zinc glycinate preparation, 1.5 μmoles of DPN, 36 μmoles of GSH, 30 μmoles of substrate, 0.1 M sodium pyrophosphate to 3.0 cc. (pH 9.2). Chicken liver was the enzyme source.

calcium phosphate gel. This procedure constitutes Step 2 of the enzyme preparation. The relative adsorption of the two enzymes on the gel is dependent on the pH, as is shown by the data given in Table I. In the region of neutrality the acetaldehyde dehydrogenase is nearly completely adsorbed on the gel while the majority of the formaldehyde dehydrogenase remains in solution. Any adsorbed formaldehyde and acetaldehyde dehydrogenase may be removed from the gel by suspending it in a mildly alkaline environment. In the standard enzyme preparation method a pH of 7.1 to 7.2 is employed to assure a supernatant fluid essentially free of the acetaldehyde enzyme, even though some loss of formaldehyde dehydrogenase is thus entailed.

Reduced glutathione has been a component in all of the enzyme assay systems to this point. Its presence as a cofactor is required, however, only

for the formaldehyde dehydrogenase system. This fact is shown in the data presented in Fig. 3, where acetaldehyde and formaldehyde dehydro-

TABLE I

Summary of Calcium Phosphate Gel Fractionation Experiments

Assay system was a measurement of the rate of DPN reduction.

Run No.	Volume gel per volume enzyme*	pH of gel suspension	Nature of supernatant	Per cent of total activity in supernatant	
				CH_2O	CH_3CHO
1	1	7.3	1st supernatant	65	6
		7.8	1st eluate	10	0
		8.5	2nd "	25	93
2	1	8.0	1st supernatant	82	36
3	1	7.8	1st "	79	26
		8.5	1st eluate	21	74
4	2	7.6	1st supernatant	65	6
		8.0	1st eluate	18	13
		8.2	2nd "	10	33
		9.0	3rd "	7	18

* The enzyme was a zinc glycinate preparation of chicken liver.

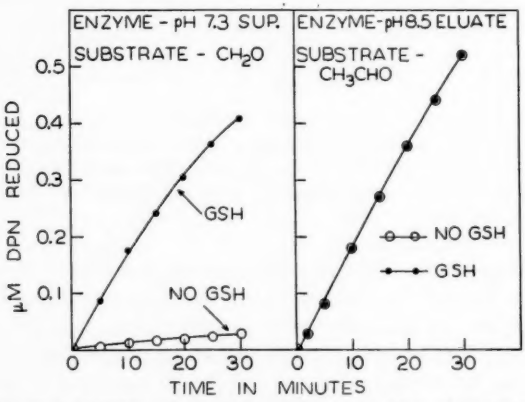


FIG. 3. The effect of GSH on formaldehyde and acetaldehyde oxidation. System, aerobic, temperature 23–26°, 30 μmoles of substrate, 1 μmole of DPN, 10 μmoles of GSH (where indicated), 0.1 M sodium pyrophosphate to 3.0 cc. (pH 9.2). Enzyme preparations as described in Table I: formaldehyde dehydrogenase, a supernatant fluid, pH 7.3, of calcium phosphate gel treatment; acetaldehyde dehydrogenase, an eluate, pH 8.5, of the calcium phosphate gel treatment. Chicken liver was the enzyme source.

genase preparations, obtained by the calcium phosphate gel treatment, have been employed as the enzyme source. It is evident from these data

that, while GSH has no effect on the acetaldehyde dehydrogenase system, the formaldehyde dehydrogenase system is essentially inactive in the absence of this cofactor.

A summary of the steps of a typical beef liver preparation appears in Table II. The resulting acetone precipitate in Step 3, a preparation essentially free of alcohol and acetaldehyde dehydrogenase activities, was used in all of the remaining studies on the formaldehyde dehydrogenase system reported below.

TABLE II

Protocol of Typical Beef Liver Preparation

Step No.	Volume or weight of enzyme preparation	Total protein	Spectrophotometric assay of DPN reduction,* μmoles $DPN_{red.}$ per mg. protein per 30 min.		
			Ethanol	Acetaldehyde	Formaldehyde
	cc.	*gm.*			
1	730	12.5	0.028	0.113	0.094
2	700	5.3	0.008	0.014	0.133
	gm.				
3	3.3	1.85	0.002	0.004	0.370†

* System, aerobic; temperature 23–26°; 2 μmoles of DPN, 2 μmoles of CH_2O, 2 μmoles of GSH, 0.1 M sodium pyrophosphate (pH 8.0).

† This specific activity varied as much as ±20 per cent in a number of preparations; however, with extreme care this variation can be reduced considerably.

Properties of Beef Liver Formaldehyde Dehydrogenase

pH Optimum—The pH optimum for formaldehyde oxidation in a system containing DPN, GSH, substrate, phosphate or pyrophosphate buffer, and enzyme was 7.9 to 8.2 for a 30 minute incubation period (Fig. 4). All assays were run in anaerobic optical cells in order to prevent autoxidation of GSH at the more alkaline pH values (11). After incubation, the pH of the contents of all tubes was determined with a Beckman pH meter and total sulfhydryl content assayed by the titrimetric method of Woodward and Fry (12). In all cases at least 85 per cent of the added amount of GSH was present after incubation, indicating that no appreciable oxidation of GSH had occurred.

A pH of 8.0 was used in all further experiments. At this pH autoxidation of GSH in the normal aerobic assay system is extremely slow: about 85 per cent of the GSH added to either an aerobic or an anaerobic system was still present in the reduced form after a 1 hour incubation and the rates of formaldehyde oxidation were the same. This fact is quite important, since oxidized glutathione is totally inactive as a substitute for reduced GSH in the formaldehyde dehydrogenase system.

Effects of Dialysis, Various Buffers, and Aging—Solutions of beef liver acetone precipitate (10 mg. per ml.) were dialyzed against 100 volumes of distilled water at 5°. The water was changed every 3 hours for a 12 hour period. No appreciable loss in enzyme activity was observed. The dialyzed enzyme was also assayed in the following buffer systems: pyrophosphate, phosphate, glycylglycine, citrate, glycine, and borate, all at pH 7.90 ± 0.10. The relative values of activity in these buffers in the order named were 1.0, 1.0, 0.9, 0.8, 0.6, and 0.5. Consequently, either pyrophosphate or phosphate buffers were used exclusively in the remaining experiments.

The dissolved acetone precipitate lost no activity on standing at 5° in air for at least 4 days in the presence or absence of EDTA. The enzyme is completely inactivated by heating it at 72° for 5 minutes.

Coenzyme and Substrate Specificity—The formaldehyde dehydrogenase system is quite specific for DPN. No appreciable reduction of TPN occurs. Acetaldehyde, propionaldehyde, isobutyraldehyde, and 3-phosphoglyceraldehyde are not attacked in this system. In all cases, when formaldehyde was added to the system after incubation with one of the other aldehydes, the usual formaldehyde activity curve was obtained; therefore, the other aldehydes also do not inhibit the formaldehyde dehydrogenase system.

Establishment of Identity of Reduced Glutathione As Cofactor—The results so far presented have indicated that the addition of reduced glutathione is necessary for the oxidation of formaldehyde by DPN. However, the glutathione used in these experiments was a commercial sample obtained from natural sources. Thus, there existed the possibility that the active cofactor is not glutathione itself but some other compound which is contained in small amounts in the glutathione sample. It was therefore important to establish conclusively whether glutathione itself was a cofactor in this oxidative process. This has been done in two ways.

First, a sample of synthetic glutathione has been employed. This was kindly supplied by Dr. Vincent du Vigneaud. It was found to give identical activation on a weight basis with that shown by several different commercial samples of glutathione.

Another approach was the use of the enzyme which specifically acts upon the γ-glutamyl peptide link of glutathione to split off cysteinylglycine (*cf.* (13)). If GSH is a specific cofactor in the formaldehyde dehydrogenase system, preincubation of the GSH with a γ-glutamyl transpeptidase preparation should then result in its inactivation. The results of an experiment with this enzyme (kindly supplied by Mr. J. P. Revel and Dr. J. H. Kinoshita) are shown in Fig. 5. It may be seen that preincubation of GSH with the transpeptidase preparation brings about a loss of its ability to

activate the formaldehyde dehydrogenase system and that maximal activity may be restored again by the addition of GSH.

It thus seems clear that glutathione itself is acting as a cofactor in the oxidation process. There now remains the question as to whether other

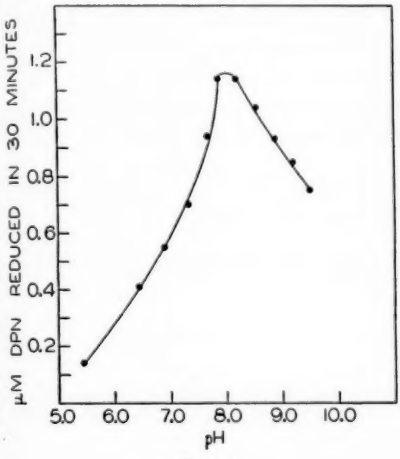


FIG. 4

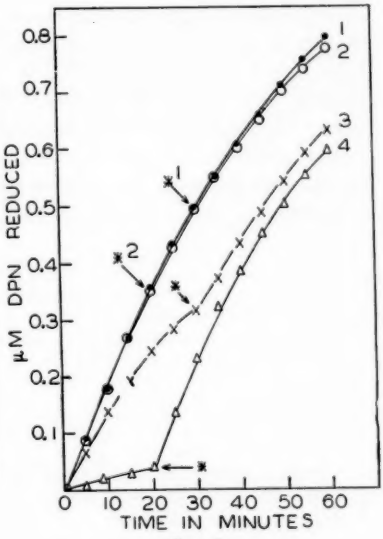


FIG. 5

FIG. 4. pH optimum curve. System, anaerobic, temperature 32°, 0.3 cc. of enzyme (3 mg. of acetone precipitate), 4 μmoles of CH_2O, 5 μmoles of GSH, 3 μmoles of DPN, 0.1 M sodium phosphate or sodium pyrophosphate to 3.0 cc. (varying pH).

FIG. 5. The effect of preincubation of GSH with γ-glutamyl transpeptidase. System, aerobic, temperature 23–26°, 0.3 cc. of enzyme (3 mg. of acetone precipitate), 0.1 cc. of 0.1 M EDTA, 1.5 μmoles of DPN, 2.0 μmoles of formaldehyde, 0.33 μmole of GSH (preincubated). The GSH was preincubated at 38° for various times, with or without transpeptidase, in 1.6 cc. of sodium phosphate buffer at pH 8.0 and under nitrogen. Curve 1, GSH preincubated for 30 minutes; Curve 2, GSH preincubated for 60 minutes; Curve 3, GSH preincubated for 30 minutes with transpeptidase; Curve 4, GSH preincubated for 60 minutes with transpeptidase. *, point of addition of another 0.33 μmole of GSH.

sulfhydryl compounds can substitute for glutathione in this capacity. The results with the hydrolysate of glutathione already indicate that one such sulfhydryl compound, cysteinylglycine, is not a substitute.

Tests of Other Sulfhydryl Compounds—Thioglycolate (shown to be 85 per cent in the reduced form by assay), ergothionine, thiolhistidine, DL-cysteine, and DL-homocysteine could not replace GSH as a cofactor. In the case

of DL-cysteine and DL-homocysteine, an inhibition of the formaldehyde dehydrogenase system containing GSH was observed, but activity could be restored by the addition of excess formaldehyde. In view of the work of Ratner and Clarke (14) demonstrating the reaction of formaldehyde with cysteine to form thiazolidine-4-carboxylic acid, it can be postulated that the inhibition observed with these two compounds results from the formation of such relatively stable cyclic complexes with formaldehyde. The inhibition would thus, in effect, be due to a lowering of substrate concentration; this is in agreement with the observation that formaldehyde reverses the inactivation observed with either sulfhydryl compound.

The addition of a CoA preparation (Pabst, 70 per cent pure, 300 Lipmann units per mg.) did produce a definite but low increase in the rate of formaldehyde oxidation over controls containing no added sulfhydryl com-

TABLE III

Assays for Reduced DPN, Formate, and Formaldehyde*

Trial No.	CH_2O added	$DPN_{red.}$ formed	CH_2O disappearing	HCOOH formed
	μmole	*μmole*	*μmole*	*μmole*
1	0.7	0.77	0.7	0.74
2	0.7	0.75	0.7	0.68

* System, aerobic; temperature 23–26°; 0.4 cc. of enzyme (3 mg. of acetone precipitate), 2.0 μmoles of DPN, 3.0 μmoles of GSH, 0.70 μmole of CH_2O, 2.0 cc. of 0.1 M sodium phosphate (pH 8.0). 70 minute incubation.

pound. However, this effect of the Pabst CoA preparation was completely abolished by preincubating the CoA with a γ-glutamyl transpeptidase preparation in the manner described for the experiments with GSH presented in Fig. 5. Assays after preincubation for CoA activity[3] by two methods (15, 16) showed that the CoA molecule was not destroyed or oxidized during this procedure. In view of these results and the fact that many purification procedures for CoA require the addition of GSH (17), we presume that the activity shown by this CoA sample is due to contamination with glutathione.

Product of Formaldehyde Oxidation and Equilibrium of Reaction—The over-all reaction in the formaldehyde dehydrogenase system was demonstrated by measuring formaldehyde disappearance, DPN reduction, and appearance of formate. In Table III are given the results of assays for reduced DPN, formate, and formaldehyde in formaldehyde dehydrogenase systems which had been incubated until the reaction stopped. The ana-

[3] We are indebted to Mr. E. Voelkel in Dr. Lipmann's laboratory for the CoA assays.

lytical results show that all of the formaldehyde was oxidized to formate with the concomitant reduction of an equivalent amount of DPN. The over-all reaction therefore appears to be

$$DPN_{ox.} + H_2O + H{-}\overset{H}{\overset{|}{C}}{=}O \xrightarrow{\text{enzyme + GSH}} HCOOH + DPN_{red.} + H^+$$

This reaction is not easily reversible. Addition of sodium formate to the formaldehyde dehydrogenase system in excess in no way affected the rate

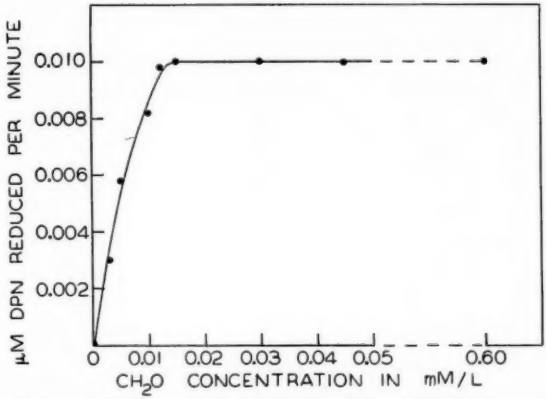


FIG. 6. Substrate concentration curve. System, aerobic, temperature 23–26°, 0.15 cc. of enzyme (1.5 mg. of acetone precipitate), 2.0 μmoles of DPN, 1.0 μmole of GSH, 0.1 M sodium phosphate to 3.0 cc. (pH 8.0), 0.066 to 2.0 μmole of formaldehyde at zero time.

of oxidation over a 90 minute period; therefore, the equilibrium for the reaction must lie far in the direction of formate.

Concentration of Formaldehyde and Glutathione Required for Maximal Activity—Spectrophotometric assays were made of the rate of oxidation at various concentrations of formaldehyde (Fig. 6). The points of the curve were obtained with systems to which 0.066 to 2.0 μmole of formaldehyde was added at zero time. The concentration of formaldehyde at any specific time during the first 5 minutes of assay was calculated by subtracting the micromoles of $DPN_{red.}$ present at that time from the total micromoles of formaldehyde added. This value for formaldehyde concentration was then plotted against the rate of formaldehyde oxidation observed during the next minute. Maximal activity was observed at formaldehyde concentrations from 6×10^{-4} to as low as 1×10^{-5} mole per liter.

The rate of formaldehyde oxidation was also determined spectrophoto-

metrically for various concentrations of GSH (Fig. 7). The minimal optimal concentration of GSH observed was 0.2 to 0.3 μmole of GSH per 3.0 cc. of reaction mixture for a 10 minute incubation and for a substrate concentration of 2.0 μmoles per 3.0 cc. In many of the assays recorded in Fig. 7, the total amount of GSH (moles) was much lower than the moles of $DPN_{red.}$ formed in a given assay period. Thus, for example, when the total amount of GSH present in the system was but 0.01 μmole, 0.09 μmole of $DPN_{red.}$ was formed in 10 minutes. Therefore, if GSH reacted in the

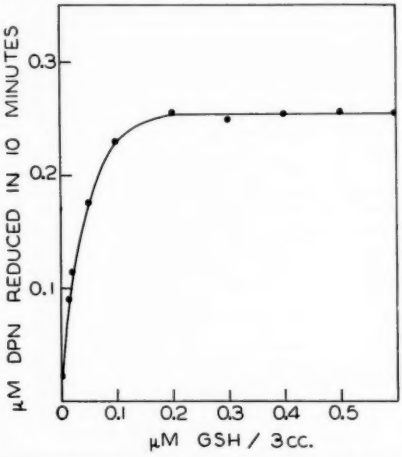


FIG. 7. Varying GSH concentrations. System, anaerobic, temperature 23–26°, 0.3 cc. of enzyme (3 mg. of acetone precipitate), 1.5 μmoles of DPN, 2.0 μmoles of formaldehyde, 0.1 M sodium phosphate to 3.0 cc. (pH 8.0), varying amounts of GSH.

formaldehyde dehydrogenase system to form an intermediate, it must have been released and reacted again in a cyclic manner.

Effect of Varying Both GSH and Formaldehyde Concentrations—The experiments recorded in Table IV show the results of a number of assays of formaldehyde oxidation in enzyme systems containing varying concentrations of GSH and formaldehyde (0 to 0.3 μmole of GSH per 3 cc. and 0 to 2.0 μmole of formaldehyde per 3 cc.). The rate of formaldehyde oxidation, as measured by DPN reduction, was recorded every minute for 10 minutes and in every case the activity curve was linear during this period. These data clearly show that the rate of formaldehyde oxidation is affected by the relative concentrations of both GSH and formaldehyde. This strongly suggests that GSH participates directly in some reaction involved in the over-all oxidation of formaldehyde to formate.

Effects of Excess Formaldehyde and Oxidized Glutathione—The addition of either formaldehyde, in concentrations as high as 10^{-2} mole per liter (30 μmoles per 3.0 cc.), or 10 μmoles of oxidized glutathione to a formaldehyde dehydrogenase system containing a minimal optimal concentration of GSH (0.3 μmole per 3.0 cc.) causes no inhibition of formaldehyde oxidation.

TABLE IV

Rate of CH_2O Oxidation with Varying Concentrations of CH_2O and GSH*

Trial No.	GSH	CH_2O	$DPN_{red.}$ per 10 min.	Per cent activity (Trial 1 taken as 100 per cent)
	μmole	*μmoles*	*μmole*	
1	0.30	2.0	0.248	100
2	0.20	2.0	0.260	109
3	0.20	0.66	0.250	101
4	0.20	0.33	0.185	75
5	0.10	2.0	0.230	93
6	0.10	0.66	0.205	83
7	0.05	2.0	0.175	71
8	0.05	2.0	0.175	71
9	0.05	0.66	0.150	61
10	0.05	0.33	0.113	46
11	0.02	2.0	0.113	46
12	0.02	0.33	0.063	25
13	0.01	2.0	0.090	36
14	0.01	0.33	0.040	16

* System, anaerobic; temperature 24° ± 1°; 0.3 cc. of enzyme (0.3 mg. of acetone precipitate), 1.5 μmoles of DPN, 0.1 M sodium phosphate to 3.0 cc. (pH 8.0). GSH was dumped from the side arm at zero time.

DISCUSSION

A specific DPN-dependent formaldehyde dehydrogenase has been identified in liver preparations and shown to be distinct from acetaldehyde dehydrogenase. The studies presented here, on some of the properties of this formaldehyde dehydrogenase system, indicate that GSH functions as an essential cofactor. In attempting to understand the rôle of GSH in this oxidative process, two rather general possibilities should be considered: (1) that GSH functions rather non-specifically to protect protein sulfhydryl groups or (2) that GSH participates directly in this reaction.

The data do not seem to be explained adequately by assuming that GSH functions non-specifically: (1) The GSH effect is quite specific: high concentrations of other sulfhydryl compounds, which might also protect protein sulfhydryl groups, could not substitute for GSH in this capacity. (2)

Large excesses of either formaldehyde or oxidized glutathione, two compounds which might inactivate protein sulfhydryl groups, do not affect the rate of formaldehyde oxidation in a system containing just an optimal concentration of reduced glutathione. (3) GSH is active in catalytic amounts and requires no preincubation with the enzyme for maximal effect. Maximal rates of formaldehyde oxidation are observed during the 1st minute following GSH addition at zero time to the remainder of the formaldehyde dehydrogenase system. (4) Finally, it is difficult to explain the observation that the rate of formaldehyde oxidation is dependent on the relative concentrations of GSH and formaldehyde on the basis of such an indirect protective mechanism.

The definite quantitative interdependence between these two components of the formaldehyde dehydrogenase system, on the other hand, does suggest that GSH functions directly, as a coenzyme, in some reaction involved in the over-all oxidation of formaldehyde to formate. The available literature on the reaction of formaldehyde with thiol groups lends support to this hypothesis. The rapid formation of hemithioacetals by the reaction of formaldehyde with thiols has been described by Ratner and Clarke (14), Schubert (18), Racker (19), and Wald *et al.* (20). Wald *et al.* have specifically shown that formaldehyde will react with the sulfhydryl group of glutathione. These findings indicate that an initial reaction, the formation of a hemithioacetal between formaldehyde and GSH, might be involved in the over-all enzymatic oxidation of formaldehyde to formate.

(1) $$\mathrm{H{-}\overset{\displaystyle H}{\overset{|}{C}}{=}O} + \mathrm{GSH} \rightleftharpoons \mathrm{H{-}\overset{\displaystyle H}{\overset{|}{C}}{-}OH{-}S{-}G};\ K_{app.} = \frac{x}{(a-x)(b-x)}$$

$(a - x)$ $(b - x)$ (x) *S*-Hydroxymethylglutathione

By making the assumption that the rate of this initial Reaction 1 in formaldehyde oxidation is not the limiting step in the over-all oxidation, it is possible to use the data of Table IV as one test for this hypothesis. If *S*-hydroxymethylglutathione is the substrate for the enzymatic oxidation of formaldehyde, the rate of formaldehyde oxidation should be directly related to the concentration of this compound. In Trials 10 and 11 (Table IV) the rates of formaldehyde oxidation were equal for the 10 minute assay period. Assuming that *S*-hydroxymethylglutathione is the actual substrate for the enzyme reaction (or reactions), the concentrations of *S*-hydroxymethylglutathione must have been equal in Trials 10 and 11. From the known concentrations of GSH (b) and formaldehyde (a) for these trials, it was possible to calculate the hypothetical concentration of *S*-

hydroxymethylglutathione (x) for Trials 10 and 11 and then a value of 1.25 for the apparent K ($K_{app.}$) of Reaction 1. The concentrations of x for the remaining trials were then calculated with this value of $K_{app.}$. When these calculated concentrations of hypothetical intermediate and the reaction rates from Table IV were plotted by a method of Lineweaver and Burk (21) (Fig. 8), the curve was similar to that which might be expected if *S*-hydroxymethylglutathione was the substrate for this enzymatic oxi-

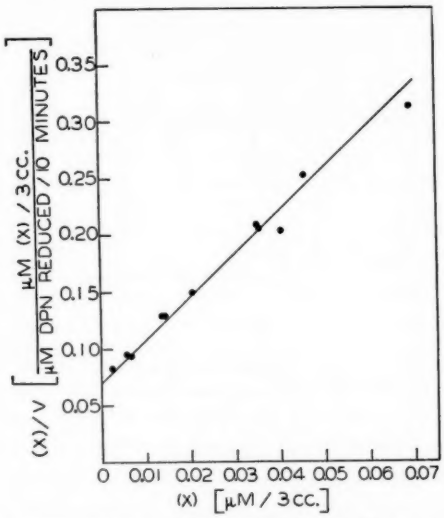
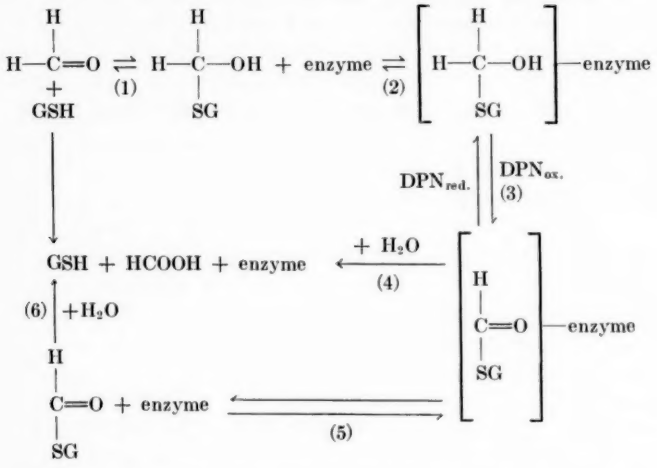


FIG. 8. Plot of concentrations of hypothetical intermediate (x) and rates of formaldehyde oxidation by a method of Lineweaver and Burk. The concentrations of x were calculated, as described in the text, for Trials 4 to 14 in Table IV, with a calculated $K_{app.}$ of 1.25 (see Reaction 1). The rate of formaldehyde oxidation for each of these trials is recorded in Table IV.

dation. As a test of the value calculated for $K_{app.}$, arbitrary values of $K_{app.}$ of 2, 5, and 0.5 were used to determine similar series of concentrations of x with each of these constants from the data in Table IV. These results, when plotted by the Lineweaver and Burk method as described above, gave a series of points for each constant that was scattered considerably further from a straight line relationship than the points shown in Fig. 8. The calculations of $K_{app.}$ and in turn the values for x therefore provide some support for the suggested initial reaction between GSH and formaldehyde, but it must be emphasized that this analysis, in itself, does not constitute proof that this particular reaction is involved in the formaldehyde dehydrogenase system.

The accompanying mechanism may, however, be postulated for the oxidation of formaldehyde in the formaldehyde dehydrogenase system.



This scheme suggests that formaldehyde reacts with GSH (Reaction 1) to form *S*-hydroxymethylglutathione which is then attached to the enzyme (Reaction 2). The product of the oxidation step (Reaction 3) might be released from the enzyme either directly as formate (Reaction 4) or as a free thiol ester (Reaction 5) which is subsequently hydrolyzed (Reaction 6) to yield formate and glutathione. It has not been possible to detect this thiol ester at any time during the reaction by its probable absorption peak in the ultraviolet region. If a thiol ester is formed in the system *in vitro*, it must be hydrolyzed rapidly, since it has been shown that glutathione can function in catalytic amounts in the formaldehyde dehydrogenase system and that formate can be isolated as the reaction product. Recent work of Stadtman (22) has indicated that formyl CoA, a similar thiol ester, is fairly stable. If the thiol ester postulated as an intermediate in the reactions given above is also fairly stable, then consideration must be given to the possibility of its enzymatic hydrolysis. With this in mind, the purest enzyme preparation was tested for thiol esterase activity with *S*-acetylglutathione, and this substrate was found to be hydrolyzed very rapidly. These facts suggest the need of further experiments to determine whether such a formyl ester of glutathione is indeed formed and, if so, whether it may participate in biological reactions involving transfer as well as hydrolysis of its formyl group.

SUMMARY

A specific DPN-dependent formaldehyde dehydrogenase has been identified in chicken and beef liver preparations, and a method for obtaining this enzyme in a form free of acetaldehyde and alcohol dehydrogenase has been presented. Reduced glutathione is a specific cofactor for this formaldehyde dehydrogenase system. The activity of this system in relation to the concentration of hydrogen ions, glutathione, and formaldehyde has been studied. On the basis of these data, a hypothesis for the mechanism of formaldehyde oxidation and the rôle of glutathione has been presented.

BIBLIOGRAPHY

1. Strittmatter, P., and Ball, E. G., *Federation Proc.*, **13,** 307 (1954).
2. Booth, V. H., *Biochem. J.*, **32,** 494 (1938).
3. Ball, E. G., *J. Biol. Chem.*, **128,** 51 (1939).
4. Gordon, A. A., Green, D. E., and Subrahmanyan, V., *Biochem. J.*, **34,** 764 (1940).
5. Racker, E., *J. Biol. Chem.*, **177,** 883 (1949).
6. Weinberger, W., *Ind. and Eng. Chem., Anal. Ed.*, **3,** 357 (1931).
7. Grant, W. M., *Ind. and Eng. Chem., Anal. Ed.*, **18,** 729 (1946).
8. Grant, W. M., *Anal. Chem.*, **20,** 267 (1948).
9. Warburg, O., and Christian, W., *Biochem. Z.*, **310,** 384 (1942).
10. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and tissue metabolism, Minneapolis, 105–109 (1949).
11. Strittmatter, P., Thesis, Harvard University (1953).
12. Woodward, G. E., and Fry, E. G., *J. Biol. Chem.*, **97,** 465 (1932).
13. Kinoshita, J. H., Thesis, Harvard University (1951).
14. Ratner, S., and Clarke, H. T., *J. Am. Chem. Soc.*, **59,** 200 (1937).
15. Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **174,** 37 (1948).
16. Barron, E. S. G., *Advances in Enzymol.*, **11,** 224 (1951).
17. Beinert, H., Von Korff, R. W., and Green, D. E., *J. Biol. Chem.*, **200,** 385 (1953).
18. Schubert, M. P., *J. Biol. Chem.*, **114,** 341 (1936).
19. Racker, E., *Biochim. et biophys. acta*, **9,** 577 (1952).
20. Wald, G., Greenblatt, C., and Brown, P. K., *Federation Proc.*, **12,** 285 (1953).
21. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
22. Stadtman, E. R., *J. Biol. Chem.*, **203,** 501 (1953).

A PYRIDINE NUCLEOTIDE-HYDROXYLAMINE REDUCTAS FROM NEUROSPORA*

By MILTON ZUCKER† and ALVIN NASON

(From the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland)

(Received for publication, August 23, 1954)

Hydroxylamine has long been postulated as an intermediate in the biological reduction of nitrate and nitrite to ammonia (1). The detection of hydroxylamine in nutrient media and tissues as well as the production of ammonia from hydroxylamine *in vivo* has been reported for a number of organisms (2–5). Recently Taniguchi and collaborators (6) found that cell-free extracts of *Bacillus pumilis* var. catalyzed the reduction of hydroxylamine to ammonia with reduced methylene blue as the electron donor. These workers implicated this enzyme, designated as hydroxylamine reductase, in the pathway of nitrate reduction. Klausmeyer and Bard (7) have also described an ammonia dehydrogenase from *Bacillus subtilis* which reversibly catalyzes the formation of DPNH[1] and ammonia.

While studying the inhibitory effect of hydroxylamine on the DPNH-linked reduction of *m*-dinitrobenzene in cell-free extracts of *Neurospora crassa*, it was found that NH_2OH greatly stimulated the endogenous rate of DPNH oxidation. This indicated that an active hydroxylamine reductase was also present in the *Neurospora* extracts.

This paper describes the partial purification and properties of *Neurospora* hydroxylamine reductase, which catalyzes the following reaction

$$NH_2OH + DPNH + H^+ \rightarrow NH_3 + H_2O + DPN^+$$

Data are also presented which indicate that a pathway of nitrate reduction in *Neurospora* involves this enzyme.

* Contribution No. 90 of the McCollum-Pratt Institute. This investigation was supported in part by a research grant (No. G-2332) from the National Institutes of Health, United States Public Health Service. A preliminary report of this work was given at the Federation meetings in April, 1954 (Zucker, M., and Nason, A., *Federation Proc.*, **13**, 328 (1954)).

† Postdoctoral Fellow of the McCollum-Pratt Institute. Present address, Connecticut Agricultural Experiment Station, New Haven, Connecticut.

[1] The following abbreviations are used: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide or riboflavin phosphate; ADH, crystalline yeast alcohol dehydrogenase.

XUM

Materials and Methods

Culture Conditions and Preparation of Cell-Free Extracts—N. crassa (wild type 5297a) was grown on 125 ml. of a modified Fries basal medium[2] in 500 ml. Erlenmeyer flasks at 30°. Micronutrient deficiencies of zinc, iron, manganese, and magnesium were obtained by omitting these elements from the media, and growth was measured as described previously (8). Molybdenum and copper deficiencies were produced by use of a copper sulfide precipitation method already described (9).

Crude, cell-free extracts were prepared from 4 to 5 day-old mycelial mats. The mats were collected on a Büchner funnel, washed with distilled water, and frozen 1 to 3 hours at −15°. The frozen mats were coarsely powdered in a cold mortar and pestle, homogenized with a Ten Broeck glass homogenizer at 0°, and centrifuged at 3000 × *g* for 20 minutes at 0°. The resulting cell-free supernatant solution contained 85 per cent or more of the hydroxylamine reductase activity in the homogenate.

Cofactors and Other Substances—DPN of 90 per cent purity was obtained from the Pabst Laboratories. TPN of 90 per cent purity was prepared from DPN with DPN kinase from pigeon liver by the method of Wang *et al.* (10), and was kindly supplied by Dr. Nathan O. Kaplan. DPNH was prepared enzymatically by the method of Pullman *et al.* (11), and TPNH enzymatically by use of the pig heart isocitric enzyme (12), as well as chemically by reduction with hydrosulfite (13). FAD of 40 per cent purity was obtained from the Sigma Chemical Company. The concentration of FAD, dissolved in distilled water, was determined spectrophotometrically with 1.13×10^7 sq. cm. mole^{-1} as the extinction coefficient at 455 mμ (14). FMN was obtained from the Nutritional Biochemicals Corporation. A boiled pig heart extract was prepared by grinding acetone-dried pig heart in 7 times its weight of 0.1 M phosphate buffer at pH 7.5, centrifuging the homogenate, and boiling the supernatant fluid for 5 minutes. After recentrifugation the boiled supernatant fluid was stored at −15° in the dark. Crystalline alcohol dehydrogenase from yeast was prepared according to Racker (15). Solutions of $NH_2OH \cdot HCl$ (Eastman Kodak Company) were prepared daily and contained no nitrite.

Determinations—Hydroxylamine was determined according to Csaky's modification (16) of the Blom procedure. Hydroxylamine was oxidized to nitrite by iodine and the nitrite determined colorimetrically by the diazo coupling procedure. To a solution containing 0.02 to 0.2 μmole of NH_2OH were added 1 ml. of 1 per cent sulfanilic acid (in 30 per cent acetic acid)

[2] Per liter, sodium tartrate 5 gm., NH_4NO_3 3 gm., KH_2PO_4 3 gm., $MgSO_4 \cdot 7H_2O$ 0.5 gm., NaCl 0.1 gm., $CaCl_2$ 0.1 gm., sucrose 20 gm., biotin 5 γ, sodium tetraborate 8.8×10^{-5} gm., $ZnSO_4 \cdot 7H_2O$ 8.8×10^{-3} gm., $CuCl_2$ 2.7×10^{-4} gm., $MnCl_2 \cdot 4H_2O$ 7.2×10^{-5} gm.

and 0.5 ml. of 1.3 per cent I_2 solution (in glacial acetic acid). After 5 minutes, the excess I_2 was destroyed by the addition of 1 ml. of 2 per cent sodium arsenite. 1 ml. of aqueous 0.02 per cent α-naphthylethylenediamine dihydrochloride was added and the reaction mixture diluted to 10 ml. with water. After $\frac{1}{2}$ hour the color was measured with a Klett-Summerson colorimeter by using a No. 54 filter. Under the above conditions 0.05 μmole of NH_2OH gave a reading of 100 Klett units, and results were linear to 0.2 μmole of NH_2OH.

NH_3 was determined by the Conway microdiffusion technique (17) in conjunction with the phenol-hypochlorite test for ammonia, as modified by Russell (18). A 2.0 ml. sample containing 0.5 to 20 γ of NH_3 was transferred to the outer well of a sealed microdiffusion unit, followed by addition of 1 ml. of saturated K_2CO_3. Diffusion of ammonia into 1.5 ml. of 0.01 N HCl contained in the center well was complete after 75 to 90 minutes at room temperature. A 0.05 ml. aliquot of acid was then removed and assayed for ammonia with the phenol-hypochlorite reagents. When the enzymatic production of ammonia was being determined, KCN at a final concentration of 10^{-4} M was added previous to the saturated K_2CO_3 in order to stop the enzymatic reaction.

Under the above conditions, ammonia added to an enzymatic reaction mixture could be recovered quantitatively. In a test volume of 10 ml., 4.1 γ of NH_3 gave a reading of 100 units on a Klett-Summerson colorimeter with a No. 66 filter.

Standard Assay of Hydroxylamine Reductase—Enzyme activity was determined spectrophotometrically by following the change in extinction at 340 mμ upon oxidation of DPNH. All measurements were made with a Beckman model DU spectrophotometer with cuvettes having a light path of 1 cm. The reaction was started by addition of 0.1 ml. of 0.4 M $NH_2OH \cdot HCl$ to a cuvette containing 2.6 ml. of 0.1 M sodium pyrophosphate buffer at pH 8.0, 0.05 ml. of 6×10^{-3} M DPNH, 0.05 ml. of boiled pig heart or 1.5×10^{-5} M FAD, and 0.2 ml. of enzyme solution, to give a total volume of 3 ml. In order to determine the endogenous rate of DPNH oxidation, the change in extinction at 340 mμ was measured at 30 second intervals for 2 to 3 minutes prior to addition of substrate. At 45 seconds after the addition of hydroxylamine, measurements of the change in optical density were begun and continued at 15 or 30 second intervals, depending on the rate of decrease in extinction, for 2 minutes. A unit of hydroxylamine reductase is defined as that amount of enzyme which causes an initial rate of change in extinction ($-\Delta E_{340}$) of 0.001 per minute calculated from the 1 to 2 minute readings, corrected for the endogenous rate of DPNH oxidation. The proportionality between enzyme concentration and initial rate of DPNH oxidation under these conditions is shown in Fig. 1. Specific

activity is expressed as units per mg. of protein. Protein was determined by the method of Lowry *et al.* (19) except in deficiency experiments in which the biuret procedure of Robinson and Hogden (20) was employed.

Results

Purification of Enzyme—All steps of the fractionation below were carried out at 0–4°, and precipitates were centrifuged at approximately 3000 × *g* at 0°. The saturated ammonium sulfate solutions used were previously

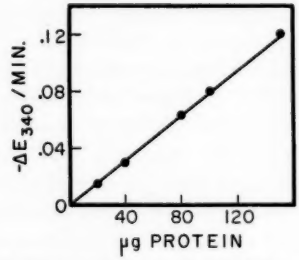


FIG. 1. Effect of enzyme concentration on the initial rate of reaction. Conditions of standard assay; enzyme purity, 800 units per mg. of protein.

TABLE I

Summary of Purification Procedure

Fraction No.		Total volume, ml.	Unit per ml., thousands	Total units, thousands	Protein, mg. per ml.	Specific activity, units per mg.	Per cent recovery
1	Crude extract	100	0.20	20	3.4	56	
2	$(NH_4)_2SO_4$ fraction	45	0.40	18	2.1	190	90
3	$Ca_3(PO_4)_2$ gel eluate	20	0.50	10	1.4	360	50

adjusted to pH 7.0 to 7.3 with concentrated NH_4OH. To 100 ml. of crude extract (Fraction 1, Table I), 83 ml. of saturated ammonium sulfate were added, making the mixture 45 per cent saturated. After standing for 5 minutes, the precipitate was removed by centrifugation, and the supernatant solution was brought to 65 per cent saturation by addition of 104 ml. more of ammonium sulfate solution. After 10 minutes the resulting precipitate was collected by centrifugation and dissolved in 40 ml. of cold distilled water. The dissolved precipitate (Fraction 2) contained 90 per cent of the original activity with a more than 3-fold increase in purity.

Fraction 2 (45 ml.) was treated with 20 ml. of calcium phosphate gel (21) containing 20 mg. per ml., dry weight. After intermittent stirring for 15

minutes, the gel precipitate was collected by centrifugation and eluted by suspending in 20 ml. of cold 0.5 M K_2HPO_4 buffer for 15 minutes. The supernatant solution obtained from subsequent centrifugation (Fraction 3) contained 50 per cent of the activity present in the original extract and represented a 6-fold purification. This fraction or the similarly prepared fractions were used as a source of enzyme in most experiments. The specific activity of these final fractions varied from 200 to 1000 units, depending upon the initial concentration of the enzyme in the crude extract.

Preparations have been obtained which had turnover numbers of up to 250 moles of TPNH oxidized per minute per mole of enzyme, assuming a molecular weight for the enzyme of 100,000.

Fraction 3 also contains a pyridine nucleotide-nitrite reductase (12, 22) which parallels hydroxylamine reductase activity under a variety of conditions, the ratio of the two activities ranging to 2½-fold. However, partially purified protein fractions have been prepared from the wild type strain 146 of *N. crassa*, which contains hydroxylamine reductase activity but no nitrite reductase activity, indicating that these are two different enzymes. Fraction 3 also contained a system capable of reducing *m*-dinitrobenzene to *m*-nitroaniline.[3] However, this system can be prepared free of hydroxylamine reductase from extracts of mycelia grown on NH_4Cl as a sole source of nitrogen.

Stability of Enzyme—Hydroxylamine reductase is most stable between pH 7.5 to 9.0, losing its activity slowly over several weeks at −15°. All activity is lost if the enzyme remains at 4° overnight or if it is heated to 50° for 5 minutes. It can be dialyzed against a 3×10^{-3} M sodium pyrophosphate, pH 8.0, and 10^{-3} M cysteine solution for at least 2 hours, losing less than half of its activity during this time. However, in most instances there is a complete loss of activity after 1 hour if 10^{-2} M phosphate, pH 8.0, is used as the dialyzing solution. This loss of activity upon dialysis cannot be restored by addition of boiled enzyme, boiled pig heart extract, FAD, or FMN (10^{-6} M final concentration). Ferrous, ferric, zinc, calcium, molybdate, cupric, manganous, borate, or magnesium ions at a final concentration of 10^{-4} M do not restore activity.

pH Optimum—The effect of pH on enzymatic activity is shown in Fig. 2. There is maximal enzyme activity between pH 7.5 and 8.5 when pyrophosphate or tris(hydroxymethyl)aminomethane is used as the buffer. Little or no activity is obtained with phosphate buffer unless 10^{-3} M Versene is included in the reaction mixture.

Substrate Affinity—The effect of hydroxylamine concentration on the reaction rate is shown in Fig. 3. The dissociation constant (K_m) for hy-

[3] Zucker, M., and Nason A., in preparation.

droxylamine, calculated from the Lineweaver-Burk (23) plot of these data (Fig. 3), is 3.8×10^{-3} M. This concentration is several orders greater than hydroxylamine levels which are toxic to *Neurospora* and other tissues.

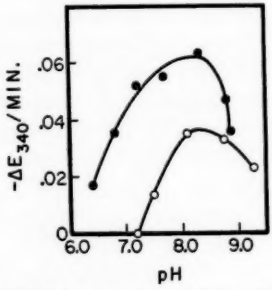


FIG. 2. Effect of pH on the initial rate of reaction. Conditions of standard assay. 0.1 M buffers used throughout and pH values shown were determined at the end of the reaction. 350 γ of enzyme (specific activity = 200) were used in each reaction mixture. ●, sodium pyrophosphate buffer. ○, Tris(hydroxymethyl)aminomethane buffer.

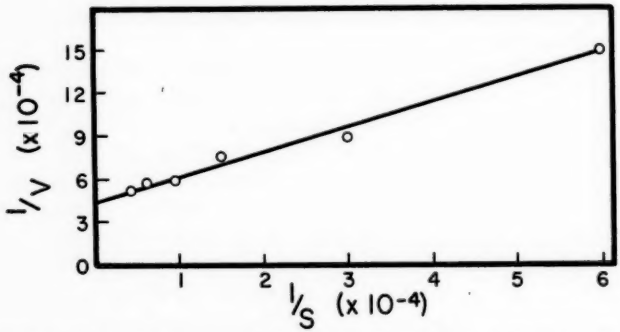


FIG. 3. Effect of NH_2OH concentration on the initial rate of reaction. Conditions of standard assay; 270 γ of enzyme (specific activity = 450) were used in each mixture. Lineweaver-Burk plot (23) of data. S = substrate concentration in moles per liter; V = the initial rate of DPNH oxidation in moles per liter per minute.

Other nitrogenous compounds were tested as substrates but no other substances beside nitrite and dinitrobenzene (see above) stimulated the oxidation of DPNH. *O*-Methylhydroxylamine, hydrazine, nitrophenylhydroxylamine, and salicylhydroxamic acid,[4] which were tested at a final concentration up to 1.3×10^{-2} M, could not replace hydroxylamine as substrate.

[4] Kindly supplied by Dr. Julius Lowenthal, University of Saskatchewan.

Reduced Pyridine Nucleotides As Electron Donors—The effect of DPNH and TPNH concentrations on the rate of reaction is shown in Fig. 4. The K_m for DPNH and TPNH, calculated from the Lineweaver-Burk (23) plots (Fig. 4), is 0.7×10^{-4} M and 1×10^{-4} M, respectively. There is no non-enzymatic oxidation of the nucleotides in the presence of NH_2OH.

Calculations of the theoretical maximal velocities from the Lineweaver-Burk plots indicate that the maximal rate attained with TPNH is almost twice that of DPNH. TPNH prepared chemically by the reduction of TPN with hydrosulfite inhibited the reaction. The degree of inhibition varied with the preparation. This inhibition is probably due to the pres-

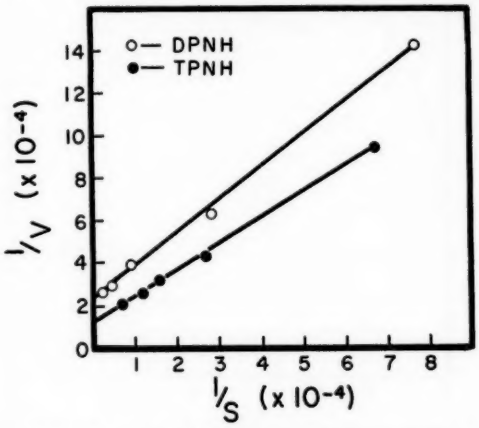


FIG. 4. Effect of pyridine nucleotide concentration on enzyme activity. Conditions of standard assay. 200 γ of enzyme (specific activity = 650) were used in each assay. Lineweaver-Burk plot (23) of data. S = substrate concentration in moles per liter; V = the initial rate of DPNH oxidation in moles per liter per minute.

ence of sulfite originating from hydrosulfite. Sulfite at 1.6×10^{-3} M final concentration completely inhibits the enzyme.

Flavin Requirement—It was observed that boiled pig heart extract stimulated the rate of DPNH oxidation 2- to 3-fold in the presence of hydroxylamine. The stimulatory effect of this extract could be replaced completely by 5×10^{-7} M final concentration of FAD. FMN had no effect, as shown in Fig. 5. The enzyme was not completely inactive in the absence of a flavin source, suggesting that some flavin was still bound to the enzyme. Although FAD alone stimulates the activity, it is not certain that this flavin is the natural prosthetic group. FAD can be detected by fluorometric analysis (24) in the boiled supernatant fluid of the enzyme, as well as by the D-amino acid oxidase procedure as described previously (12).

Evidence for Metal Constituent—Hydroxylamine reductase is very sensitive to KCN and is inhibited approximately 50 per cent at 5×10^{-6} M final concentration. A number of other chelating agents at higher concentration inhibit the enzyme (Table II), suggesting the presence of a metal as an active constituent in the system. Attempts to identify the metal or metals involved have proved unsuccessful thus far. However, extracts

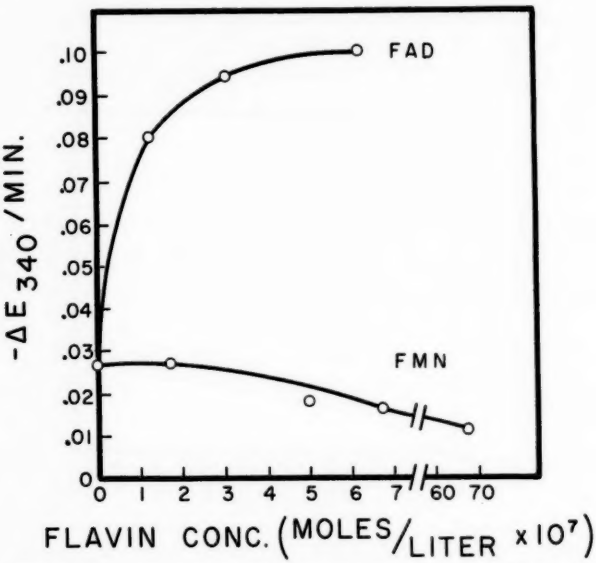


FIG. 5. Effect of flavin on the initial rate of reaction. Conditions of standard assay. 230 γ of enzyme (specific activity = 460) were used in each mixture. Initial rate ($-\Delta E_{340}$ per minute) obtained with boiled pig heart in place of FAD = 0.110.

of mycelia grown on media deficient in zinc, iron, or copper exhibit markedly lower specific activities.

A 5 minute preincubation of Zn^{++}, Cu^{++}, Fe^{+++}, Fe^{++}, Mn^{++}, Mg^{++}, Ca^{++}, $MoO_4^{=}$, $BO_3^{=}$, or Co^{++} at a final concentration of 10^{-4} M with a purified active preparation of hydroxylamine reductase as well as with a dialyzed enzyme preparation failed to increase the activity of the enzyme. Preincubation with Cu^{++} at 10^{-4} M concentration resulted in a 70 per cent loss of enzyme activity. Molybdate ions increased the endogenous DPNH oxidation but inhibited the hydroxylamine reductase activity.

Products and Stoichiometry of Reaction—The disappearance of the 340 mμ absorption of DPNH on addition of hydroxylamine to a complete re-

action mixture is shown in Fig. 6. At the end of the reaction, a system such as alcohol and alcohol dehydrogenase capable of regenerating DPNH from DPN was added. As shown, better than 90 per cent of the original absorption at 340 mμ was recovered, demonstrating that DPN is a product of the reaction.

Ammonia As Product—Ammonia was identified as a product of the enzymatic reaction by means of the Conway microdiffusion technique in conjunction with the phenol hypochlorite test for ammonia, as already described under "Materials and methods." The high concentration of

TABLE II

Inhibition of Hydroxylamine Reductase by Metal-Binding Agents; Per Cent Inhibition of Initial Rate of Reaction

Inhibitor	Final concentration		
	10^{-2} M	10^{-3} M	5×10^{-4} M
KCN	100	100	50
Salicylaldoxime	66	0	
NaN_3	65	0	
Potassium ethyl xanthate	50	0	
Na diethyl dithiocarbamate	30		
O-Phenanthroline	30		
8-Hydroxyquinoline	30		
α,α-Dipyridyl	10		
Thiourea	0		
KCNS	0		

Conditions of standard assay. All chelating agents added in 0.1 M pyrophosphate at pH 8.0. 200 γ of enzyme (specific activity = 500).

ammonia in the purified enzyme, as a result of the ammonium sulfate fractionation step, was almost completely removed by dialysis for 1 to 2 hours at 4° against a large volume of 3×10^{-3} M pyrophosphate, pH 8.0, and 10^{-3} M cysteine hydrochloride in triply distilled water. Approximately one-half of the enzyme activity was lost by this treatment.

In the time-course of enzymatic ammonia production (Fig. 7), all the values are corrected for endogenous ammonia by using the complete reaction mixture stopped by addition of KCN at zero time as the blank. Up to 0.5 μmole of ammonia was liberated non-enzymatically from the dialyzed enzyme and a similar quantity was produced chemically from the NH_2OH. If DPNH, hydroxylamine, or the enzyme is omitted from the reaction mixture, no ammonia above the endogenous level is produced. The cessation of ammonia production after 30 minutes is apparently due to inac-

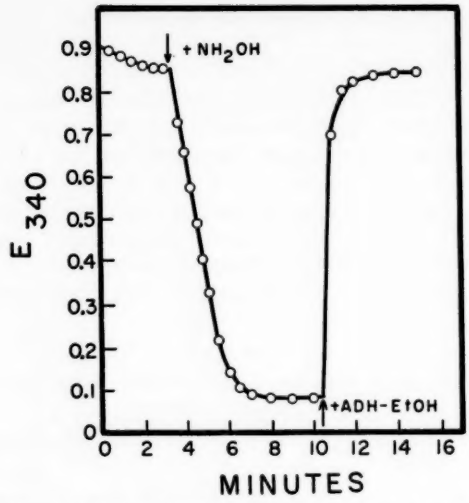


FIG. 6. Time-course of hydroxylamine reductase reaction. Conditions of standard assay. At the first arrow, 0.1 ml. of 0.4 M $NH_2OH \cdot HCl$ was added. At the second arrow, 0.05 ml. of yeast alcohol dehydrogenase (3 γ of protein) and 0.05 ml. of 95 per cent ethyl alcohol (EtOH) were added. 400 γ of enzyme (specific activity = 800) were used in the assay.

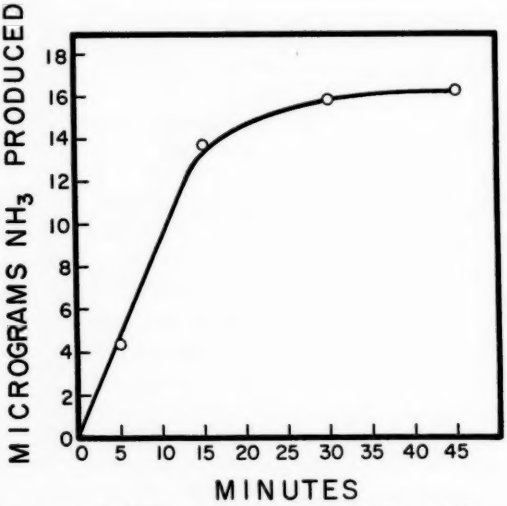


FIG. 7. Time-course of NH_3 production. The reaction mixture contained 1.2 μmoles of DPNH, 2×10^{-6} μmole of FAD, 0.2 ml. of enzyme (300 γ of protein), and 0.1 M pyrophosphate at pH 8.0 to 2 ml.

tivation of the enzyme, for ammonia production resumes again if more enzyme is added.

Stoichiometry—The stoichiometry of the hydroxylamine reductase reaction was determined by measuring hydroxylamine disappearance and

TABLE III

Stoichiometry of Hydroxylamine Reductase Reaction from Enzymatic Rates of DPNH Oxidation and Hydroxylamine Disappearance

Experiment No.		DPNH oxidized	Reduced NH_2OH	Ratio DPNH:NH_2OH
	min.	*μmole*	*μmole*	
1	0	0	0	
	15	0.12	0.09	1.33
	30	0.20	0.19	1.05
2	0	0	0	
	30	0.18	0.17	1.07
	180*	0.42	0.48	0.88
3	0	0	0	
	15	0.67	0.53	1.26
	60	0.74	0.79	0.94
4	25	0.76	0.75	1.01

Experiment 1, the reaction mixture contained 1.2 μmoles of DPNH, 0.05 ml. of boiled pig heart, and 0.5 ml. of enzyme (500 γ of protein), 1 μmole of $NH_2OH \cdot HCl$, and 0.2 M pyrophosphate at pH 8.0 to make a total volume of 1 ml. *Experiment 2*, as in Experiment 1, except that 1.8 μmoles of DPNH and 0.2 ml. of enzyme were used. *Experiment 3*, the reaction mixture contained 1.5 μmoles of DPNH, 2×10^{-6} μmole of FAD, 1 ml. of enzyme (1800 γ of protein), and 2.5 μmoles of $NH_2OH \cdot HCl$ and 0.2 M pyrophosphate at pH 8.0 to a volume of 1 ml. *Experiment 4*, the same as Experiment 3, except that 1 μmole of DPNH was used. KCN at a final concentration of 10^{-4} M was added to all aliquots used for DPNH and NH_2OH assay immediately after removal to stop the reaction. In all experiments, DPNH values were corrected for endogenous DPNH oxidation by use of reaction mixtures from which NH_2OH was omitted. Low hydroxylamine concentrations were used in the reaction mixtures to avoid excessive dilutions otherwise necessary for the hydroxylamine assay described in the text.

* After 30 minutes 1 ml. more of enzyme was added and reaction was allowed to incubate until 180 minutes.

DPNH oxidation with undialyzed enzyme preparations, and NH_3 production and DPNH oxidation with dialyzed enzymes. DPNH oxidation was determined from the change in the 340 mμ absorption during incubation of the complete reaction mixture after subtraction of the low endogenous oxidation value observed in the absence of substrate. The extinction coefficient of 6.24×10^6 sq. cm. $\times$ $mole^{-1}$ at 340 mμ (25) was used to compute DPNH concentration. In several hydroxylamine experiments, DPNH oxidation was also determined from the increase in 340 mμ absorp-

tion at the end of the reaction after addition of alcohol and alcohol dehydrogenase, thus confirming the value obtained directly.

In Table III is a mole for mole relationship between the NH_2OH disappearance and DPNH oxidation. No hydroxylamine disappeared after an hour's incubation in a complete reaction mixture inhibited at zero time with cyanide.

TABLE IV

Stoichiometry of Hydroxylamine Reductase Reaction from Enzymatic Rates of DPNH Oxidation and Ammonia Formation

Experiment No.		DPNH oxidized	NH_3 produced	Ratio, DPNH: NH_3
	min.	*μmole*	*μmole*	
1	0	0	0	
	30	0.52	0.58	0.90
	30	0.53	0.63	0.84
2	0	0	0	
	4	0.17	0.20	0.85
	8	0.23	0.26	0.88
	25	0.41	0.33	1.24

Experiment 1, the reaction mixture contained 1 μmole of DPNH, 2×10^{-6} μmole of FAD, 0.4 ml. of enzyme (420 γ of protein), 40 μmoles of $NH_2OH \cdot HCl$, and 0.2 M pyrophosphate at pH 8.0 to a total volume of 3 ml. 2 ml. aliquots were removed for NH_3 determinations. *Experiment 2*, the reaction mixture contained 1.2 μmoles of DPNH, 2×10^{-6} μmole of FAD, 0.2 ml. of enzyme (240 γ of protein), 10 μmoles of $NH_2OH \cdot HCl$ and 0.2 M pyrophosphate to 2 ml. Reaction run in Conway diffusion units. NH_3 determinations are described in the text. In all experiments DPNH values were corrected for endogenous DPNH oxidation by use of reaction mixtures from which NH_2OH was omitted. Endogenous NH_3 was corrected for as described in the text.

A similar 1:1 ratio between NH_3 production and DPNH oxidation is shown in Table IV. These data support the following equation for the reaction

$$NH_2OH + DPNH + H^+ \rightarrow NH_3 + H_2O + DPN^+$$

Adaptation Experiments—The effects of various nitrogen sources on the production of hydroxylamine reductase are shown in Table V. Activity occurred only in extracts of mycelia grown in the presence of nitrate or nitrite, suggesting the adaptive nature of the enzyme. When nitrite served as the sole nitrogen source, hydroxylamine reductase was markedly lowered and could be determined only in purified preparations. When both nitrate and nitrite were added to the nutrient medium, enzyme preparations were obtained of much lower specific activity compared to nitrate alone,

indicating an inhibitory effect of nitrite. A similar effect on nitrite reductase from the same organism was also observed. No activity existed in mycelia grown on ammonium salts or alanine as the sole nitrogen source. The possibility that ammonia is inhibitory was ruled out by the high concentration of the enzyme in mycelia grown on an ammonium nitrate-containing medium. Experiments in which extracts of ammonia-grown mycelia were mixed with those of nitrate-grown mycelia indicated that there was no inhibition in the former nor an activator in the latter. Hydroxylamine was too toxic to be used as a sole nitrogen source.

TABLE V

Effect of Nitrogen Source on NH_2OH Reductase Content

Experiment No.	Nitrogen source	E_{340} per min. per ml.	Protein per ml.	Specific activity
			mg.	
I. Crude extract	KNO_3	0.660	3.5	0.19
	NH_4NO_3	0.570	4.2	0.14
	NH_4Cl	0	4.4	0
	DL-Alanine	0	4.3	0
II. Gel eluate	KNO_3	1.880	1.5	1.26
	KNO_3, KNO_2	0.780	1.1	0.71
	KNO_2	0.180	1.2	0.15
	NH_4Cl	0	1.4	0

KNO_3 medium, same as the Fries medium described in the text except that 3 gm. per liter of NH_4NO_3 were replaced by 3 gm. per liter of KNO_3. In NH_4Cl medium, NH_4NO_3 was replaced by 3 gm. per liter of NH_4Cl. In DL-alanine medium, 9 gm. per liter of the amino acid were used as an N source instead of NH_4NO_3. The KNO_2 medium contained 0.5 gm. per liter of KNO_2 instead of NH_4NO_3. 3 gm. per liter of KNO_3 and 0.5 gm. per liter of KNO_2 were added as the N source in the KNO_3, KNO_2 medium. Mycelia were grown for 4 days and extracted as described in "Materials and methods." Activity was determined under conditions of the standard assay. Gel eluates (Fraction 3) used in Experiment 2 were obtained by fractionating each extract as described in the text.

DISCUSSION

Taniguchi and collaborators (6) have shown that cell-free extracts of *B. pumilis* can reduce nitrate, nitrite, and hydroxylamine to ammonia in the presence of reduced methylene blue as an electron donor. They postulated that these systems represent the major pathway of nitrate reduction *in vivo*.

Both a pyridine nucleotide-linked nitrate and nitrite reductase have been described in *Neurospora* (12, 22). These, together with the present data, suggest that a similar stepwise pathway of nitrate reduction to ammonia exists in this organism in which the pyridine nucleotides serve as natural electron donors. It has been possible to demonstrate the enzymatic con-

version of nitrite to ammonia by DPNH with the partially purified fraction described above, which contains both the nitrite and hydroxylamine reductase (22). Similar results have been obtained by using a protein fraction from soy bean leaves which also contains both enzymes (22).

The adaptation experiments furnish further support for the existence of this pathway *in vivo*. The formation of hydroxylamine reductase when nitrate but not ammonium ions alone are present in the nutrient medium suggests that nitrate ions give rise to hydroxylamine, which in turn stimulates the adaptive formation of its reductase.

The inhibitory effect of nitrite may be a general one involving the growth of the organism as well as a specific inhibition of synthesis, activity, or extractability of the enzyme. A similar inhibition *in vivo* of the production of N_2 in *Pseudomonas* has also been noted (26).

Whether the above pathway of nitrate reduction to ammonia is the principal mechanism of inorganic nitrogen assimilation in *Neurospora* is open to question. It is possible that a major pathway of nitrate metabolism exists involving the incorporation of inorganic nitrogen into an organic molecule before reduction to the amino level (27).

Silver and McElroy (28) have reported four different genetic blocks in *Neurospora* which prevent the utilization of NO_2^- as a nitrogen source. However, both nitrite reductase (28) and hydroxylamine reductase[5] have been found in all four mutant types. If these enzymes were of major importance in the utilization of nitrite, then it is difficult to understand why all of the mutants fail to grow on this nitrogen source. A possible explanation that Silver and McElroy (28) have suggested on the basis of their work with a pyridoxine mutant is that hydroxylamine is reduced only after combination with pyridoxal phosphate.

The electron carrier rôle of both flavin and metal has been indicated for the mechanism of action of nitrate reductase (29) and other enzymes such as butyryl CoA dehydrogenase (30). It seems likely that the flavin and metal components of hydroxylamine reductase will prove to act in a similar manner. From the E'_0 values at pH 7.0 of DPNH:DPN$^+$ (31) and NH_2OH:NH_3 (32), -0.3 and $+0.8$ volts, respectively, a change in free energy of approximately 47,000 calories was calculated for the hydroxylamine reductase reaction. This indicates that the reaction has a very large equilibrium constant, K, of about 10^{35}. Various attempts to reverse the reaction by use of hydroxylamine trapping reagents, increased pH, and varying concentrations of ammonia and DPN were unsuccessful. Nor did NH_4Cl decrease the initial rate of DPNH oxidation in a standard assay. This is in contrast to the results of Klausmeyer and Bard (7), who

[5] Zucker, M., and McElroy, W. D., unpublished data.

have reported the reversibility of the ammonia dehydrogenase from *B. subtilis*.

Identification of ammonia as a product of the reaction is based on its rapid diffusibility and its reaction with the phenol-hypochlorite reagents. Aliphatic amines also have this property. However, for the most part they have a considerably lower diffusion rate (17) and would not be completely absorbed within an hour at room temperature as is the enzymatic product. Furthermore, it is unlikely that the carbon skeleton of an amine would be furnished by the breakdown of protein or provided in substrate amounts by the dialyzed enzyme preparations used.

SUMMARY

A soluble enzyme system which reduces hydroxylamine to ammonia has been purified 5- to 10-fold from cell-free extracts of *Neurospora crassa*. Both DPNH and TPNH serve as electron donors in the reaction. FAD but not FMN stimulates the activity of the enzyme 2- to 3-fold and metal chelating agents inhibit the activity of the enzyme (KCN being the most effective), suggesting a metal constituent. Stoichiometry among hydroxylamine disappearance, DPNH as well as NH_3 production, and DPN formation support the following equation for the reaction

$$NH_2OH + DPNH + H^+ \rightarrow NH_3 + DPN^+ + H_2O$$

The reaction has not been reversed. The enzyme is adaptive, forming only in the presence of nitrate or nitrite but not ammonia. These data suggest that NO_3^- can give rise to hydroxylamine and subsequently to ammonia in *Neurospora*.

BIBLIOGRAPHY

1. Virtanen, A. I., and Rautanen, N., in Sumner, J. B., and Myrbäck, K., The enzymes, **2**, pt. 2, 1089 (1952).
2. Wood, J. G., *Ann. Rev. Plant Physiol.*, **4**, 1 (1954).
3. Lascelles, J., and Still, J. L., *Australian J. Exp. Biol. and Med. Sc.*, **24**, 159 (1946).
4. Lemoigne, M., de Somer, A., and Croson, M., *Compt. rend. Acad.*, **232**, 1778 (1951).
5. Lewis, D., *Biochem. J.*, **49**, 149 (1951).
6. Taniguchi, S., Mitsui, H., Toyoda, J., Yamada, T., and Egami, F., *J. Biochem.*, Japan, **40**, 175 (1953).
7. Klausmeyer, R., and Bard, R., *J. Bact.*, **68**, 129 (1954).
8. Nason, A., Kaplan, N. O., and Colowick, S. P., *J. Biol. Chem.*, **188**, 397 (1951).
9. Nicholas, D. J. D., *Analyst*, **77**, 629 (1952).
10. Wang, T. P., Kaplan, N. O., and Stolzenbach, F. E., *J. Biol. Chem.*, **211**, 465 (1954).
11. Pullman, M. E., Colowick, S. P., and Kaplan, N. O., *J. Biol. Chem.*, **194**, 593 (1952).

12. Nason, A., and Evans, H. J., *J. Biol. Chem.*, **202**, 655 (1953).
13. Colowick, S. P., Kaplan, N. O., Neufeld, E. F., and Ciotti, M. M., *J. Biol. Chem.*, **195**, 95 (1952).
14. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150 (1938).
15. Racker, E., *J. Biol. Chem.*, **184**, 313 (1950).
16. Csaky, T. Z., *Acta chem. Scand.*, **2**, 450 (1948).
17. Conway, E. J., Micro-diffusion analysis and volumetric error, London, 2nd edition (1947).
18. Russell, J. A., *J. Biol. Chem.*, **156**, 457 (1944).
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
20. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135**, 727 (1940).
21. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124**, 397 (1938).
22. Nason, A., Abraham, R. G., and Averbach, B. C., *Biochim. et biophys. acta*, **15**, 159 (1954).
23. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
24. Burch, H. B., Bessey, O. A., and Lowry, O. H., *J. Biol. Chem.*, **175**, 457 (1948).
25. Horecker, B. L., and Kornberg, A., *J. Biol. Chem.*, **175**, 385 (1948).
26. Allen, M. B., and Van Niel, C. B., *J. Bact.*, **64**, 397 (1952).
27. de la Haba, G., *Science*, **112**, 203 (1950).
28. Silver, W. S., and McElroy, W. D., *Arch. Biochem. and Biophys.*, **51**, 379 (1954).
29. Nicholas, D. J. D., and Nason, A., *Arch. Biochem. and Biophys.*, **51**, 310 (1954).
30. Mahler, H. R., *J. Am. Chem. Soc.*, **75**, 3288 (1953).
31. Burton, K., and Wilson, T. H., *Biochem. J.*, **54**, 86 (1953).
32. Latimer, W. M., and Hildebrand, J. H., Reference book of inorganic chemistry, revised edition, 474 (1940).

CONVERSION OF RADIOACTIVE GLUCOSE AND ACETATE TO TRYPTOPHAN BY AEROBACTER AEROGENES*

By MAX E. RAFELSON, Jr.

(From the Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois)

(Received for publication, September 2, 1954)

Previous studies (1, 2) have described the formation of tryptophan in *Aerobacter aerogenes* grown on acetate-1-C^{14} as the sole source of carbon. The data indicated that the carboxyl carbon atom of acetate was a major source for 3 consecutive carbon atoms of the benzene ring, 1 carbon atom of the pyrrole ring, and the carboxyl carbon atom of tryptophan. It was suggested that the labeling in the benzene ring did not reflect the direct utilization of acetate *per se*. It appeared possible that the acetate might have been converted first to 3,4-labeled glucose, and that this was a more immediate carbon source for the benzene ring. It was further suggested that the carbon atom of the pyrrole ring derived from the carboxyl carbon atom of acetate arose directly from acetate. An attempt has been made in the present communication to provide direct evidence for the above suggestion. *A. aerogenes* has been cultivated on glucose-3,4-C^{14} to determine whether the labeling sequence in the benzene ring of tryptophan would be the same as that obtained with acetate-1-C^{14}. In a second cultivation, *A. aerogenes* was grown on unlabeled glucose in the presence of acetate-1-C^{14}.

EXPERIMENTAL

The organism used was a strain of *A. aerogenes* having the characteristics previously described (1).

The growth medium was that of Cutinelli *et al.* (3), with the substitution of glucose at a concentration of 1.0 mg. per ml. for the acetate. In Experiment I (Table I) glucose-3,4-C^{14}, containing 1025 c.p.m. per mg. of C, was the only source of carbon. The labeled glucose was isolated from rat liver glycogen formed in the presence of $C^{14}O_2$. Degradation of this according to Wood *et al.* (4) showed that 98 per cent of the radioactivity was in carbon atoms 3 and 4 of the glucose molecule. Acetate-1-C^{14} (72,000 c.p.m. per mg. of C) was used in Experiment II (Table III) at a concentration of 0.5 mg. per ml. in the presence of unlabeled glucose. The bacteria were cultivated aerobically for 4 hours in 10 liters of the radioactive medium.

The procedures for the isolation of tryptophan as the azobenzene-4-

* Supported in part by a grant from the National Institutes of Health, United States Public Health Service.

sulfonate derivative (1) and for the degradation of tryptophan (2) have been described in detail. For the degradations, 1.21 gm. of tryptophan (51 c.p.m. per mg. of C) and 1.35 gm. of tryptophan (20 c.p.m. per mg. of C)

TABLE I

Isotope Content of Carbon Atoms of Tryptophan Isolated from A. aerogenes Grown on Glucose-3,4-C^{14} (Experiment I)

Series	Compound	Reaction	Tryptophan carbons*	C.p.m. per mg. C		Calculations†
				Determined	Calculated	
A	Tryptophan	Combustion	All‡	51	48	See Table III
B	"	Decarboxylation	COOH	122		
C			α		16	11A − 9D − B§
D	Skatole	Combustion	2–9, β‡	47	46	(9F + E)/9
E	3-Cl-quinoline-4-COOH	Decarboxylation	β	22		
F	3-Cl-quinoline	Combustion	2–9, 0‡	44	49	(9D − E)/9
G	5-Cl-quinolinic acid	"	2–5, 8, 9, 0‡	27	27	(5K + H + J)/7
H	" "	Decarboxylation	8	12	15	7G − 6I
I	5-Cl-nicotinic acid	Combustion	2–5, 9, 0‡	29	29	(5K + J)/6
J	" "	Decarboxylation	5	100	99	6I − 5K
K	3-Cl-pyridine	Combustion	2–4, 9, 0‡	15	9	(2L + N + U + V)/5
L	Oxalic acid	"	2, 0‡	8		
L′	" "		2	16		
M	Salicylic acid	Combustion	3–9‡	96	99	(6P + N)/7
N	" "	Decarboxylation	3	14	−6	7M − 6P
O	3,5-Di-NO_2-salicylic acid	Bromopicrin	6, 8‡	61	57	(H + S)/2
P	Phenol	Combustion	4–9‡	113	117	(3Q + 3Q′)/6
Q	Picric acid	Bromopicrin	4, 6, 8‡	44		
Q′	" "	"	5, 7, 9‡	73		
R	Trimethyl pyruvate	Decarboxylation	5, 7‡	106		
S	Pivaloyl chloride	"	6	102	110	2O − H
T			7		112	2R − J
U			4		10	3Q − 2O
V			9		7	3Q′ − 2R

* See Fig. 1 for numbering of the carbon atoms.

† Directly determined values used when possible. Only a few of the possible calculations have been indicated in this column.

‡ Mean values for each.

§ 11A − 9D − B represents (11 × 51) − (9 × 47) − 122 = 16.

were used, respectively, for Experiments I and II. The individual carbon atoms or groups of carbon atoms were converted to carbon dioxide by combustion and isolated as barium carbonate. All barium carbonate preparations were counted at infinite thickness in a gas flow counter. The probable error in counting was less than 3 per cent.

TABLE II

Compiled Values for Isotope Distribution in Tryptophan

Tryptophan carbon atom*	C.p.m. per mg. C		Per cent of total activity		
	Experiment I†	Experiment II‡	Experiment I	Experiment II	Experiment III§
COOH	122	18	22.9	8.5	25.8
α	16	4	3.0	1.8	0.2
β	22	6	4.1	2.7	1.4
2	16	144	3.0	64.3	15.5
3	14	4	2.6	1.8	0.3
4	10	4	1.9	1.8	0.0
5	100	12	18.8	5.4	10.6
6	102	10	19.1	4.5	21.6
7	112	14	21.0	6.3	25.1
8	12	4	2.3	1.8	0.4
9	7	4	1.3	1.8	0.2
Total	533	224			
Mean	48	20			
Observed mean	51	20			

* See Fig. 1 for numbering of the carbon atoms.

† Compiled from Table I. Glucose-3,4-C^{14} was the only carbon source.

‡ Compiled from Table III. Acetate-1-C^{14} and unlabeled glucose were the source of carbon.

§ Data from Rafelson (2). Acetate-1-C^{14} was the only carbon source.

The results of the various determinations of the isotope content of the tryptophan carbon atoms appear in Tables I, II, and III. The numbering of the tryptophan carbon atoms is arbitrary and is shown in Fig. 1.

DISCUSSION

The data in Table I, compiled in Table II (Experiment I), indicate that 3 consecutive carbon atoms of the benzene ring (C_5, C_6, and C_7) and the carboxyl carbon atom of tryptophan originated from carbon atoms 3 and 4 of glucose. This is similar to the labeling sequence obtained in tryptophan when *A. aerogenes* was grown on acetate-1-C^{14} as the only source of carbon (1, 2). For convenience these data are presented in Table II as Experiment III. If, as suggested (1, 2), acetate-1-C^{14} was not used directly

for the formation of the benzene ring, but was first converted to a 3,4-labeled hexose unit, glucose-3,4-C^{14} should give the same labeling sequence in the benzene ring of tryptophan as was obtained with acetate-1-C^{14}. A comparison of the compiled data in Table II, Experiments I and III, shows that as far as the benzene ring is concerned the same qualitative picture was obtained in that only C_5, C_6, and C_7 of tryptophan originated from carbon atoms 3 and 4 of glucose or the carboxyl carbon atom of acetate. With glucose-3,4-C^{14} these 3 carbon atoms were equally labeled, each containing approximately 20 per cent of the total radioactivity in tryptophan. In the experiment with acetate-1-C^{14} as the only carbon source, C_5 contained only 50 per cent of the activity found in C_6 and C_7, which were approximately equally labeled. The reason for this unequal utilization of the acetate carboxyl carbon atom is not known. While C_2 of tryptophan had an origin from the carboxyl carbon of acetate in Experiment III, it did not arise from carbon atoms 3 and 4 of glucose. This was to be expected if C_2 arose from the carboxyl carbon atom of acetate, since glucose-3,4-C^{14} should not give rise to appreciable amounts of acetate-1-C^{14} if the glucose-acetate conversion occurs in the conventional manner.

Experiment II (Table III) was designed to determine whether C_2 of the pyrrole ring of tryptophan might have a direct origin from the carboxyl carbon atom of acetate. The data in Table III (compiled in Table II) furnish some presumptive evidence to support this view. Approximately 64 per cent of the total activity in tryptophan, derived from acetate-1-C^{14} in the presence of unlabeled glucose, was localized in C_2 (Table II). The isotope content in C_5, C_6, and C_7 was considerably less in this experiment than in Experiments I and III, indicating that the acetate carboxyl carbon atom in the presence of glucose is not a major source for these carbon atoms and that they originate mainly from glucose. It is perhaps surprising that C_5, C_6, and C_7 had any origin from the acetate carboxyl in Experiment II, since Gilvarg and Bloch (5) found that acetate was not converted to glucose by actively growing *Saccharomyces cerevisiae*. However, the glucose reisolated from the medium of Experiment II was radioactive. Degradation according to Wood *et al.* (4) revealed that 95 per cent of the radioactivity was in positions 3 and 4 of the glucose molecule. Since it was not possible to determine the relative dilutions involved in the isolation of the glucose and of the tryptophan, their specific activities could not be directly compared. However, C_5, C_6, and C_7 were essentially equally labeled in this experiment, as in the experiment with glucose-3,4-C^{14}. This suggests that the labeling in these 3 carbon atoms was due to the conversion of a portion of the acetate-1-C^{14} to 3,4-labeled glucose.

The data presented in this communication are compatible with the suggestions (1, 2) that the benzene ring of tryptophan may be derived from a hexose series of reactions, while the pyrrole ring of tryptophan arises

directly from acetate. This hypothetical scheme is presented in Fig. 1. The early stages of the scheme are those proposed by Ehrensvärd and Reio (6) for the formation of the benzene ring of tyrosine. The possibility that

TABLE III

Isotope Content of Carbon Atoms of Tryptophan Isolated from A. aerogenes Grown on Acetate-1-C^{14} and Unlabeled Glucose (Experiment II)

Series	Compound	Reaction	Tryptophan carbons*	C.p.m. per mg. C		Calculations†
				Determined	Calculated	
A	Tryptophan	Combustion	All‡	20	20	See Table III
B	"	Decarboxylation	COOH	18		
C			α		4	11A − 9D − B§
D	Skatole	Combustion	2–9, β‡	22	24	(9F + E)/9
E	3-Cl-quinoline-4-COOH	Decarboxylation	β	6		
F	3-Cl-quinoline	Combustion	2–9, 0‡	23	21	(9D − E)/9
G	5-Cl-quinolinic acid	"	2–5, 8, 9, 0‡	27	26	(5K + H + J)/7
H	" "	Decarboxylation	8	4	9	7G − 6I
I	5-Cl-nicotinic acid	Combustion	2–5, 9, 0‡	30	30	(5K + J)/6
J	" "	Decarboxylation	5	12	15	6I − 5K
K	3-Cl-pyridine	Combustion	2–4, 9, 0‡	33	31	(2L + N + U + V)/5
L	Oxalic acid	"	2, 0‡	72		
L′	" "		2	144		
M	Salicylic acid	Combustion	3–9‡	9	8	(6P + N)/7
N	" "	Decarboxylation	3	4	9	7M − 6P
O	3,5-Di-NO_2-salicylic acid	Bromopicrin	6, 8‡	7	7	(H + 5)/2
P	Phenol	Combustion	4–9‡	9	8	(3Q + 3Q′)/6
Q	Picric acid	Bromopicrin	4, 6, 8‡	6		
Q′	" "	"	5, 7, 9‡	10		
R	Trimethyl pyruvate	Decarboxylation	5, 7‡	13		
S	Pivaloyl chloride	"	6	10	10	2O − H
T			7		14	2R − J
U			4		4	3Q − 2O
V			9		4	3Q′ − 2R

* See Fig. 1 for numbering of the carbon atoms.

† Directly determined values used when possible. Only a few of the possible calculations have been indicated in this column.

‡ Mean values for each.

§ 11A − 9D − B represents (11 × 20) − (9 × 22) − 18 = 4.

C_3 and C_4 fragments, derived from glucose, lead to the formation of C_7 sugars has been reported by Horecker *et al.* (7–10) and Axelrod *et al.* (11). Gilvarg and Bloch (12) and Ehrensvärd (6, 13) have discussed the possible rôle of C_7 sugars in the biosynthesis of aromatic rings. Robinson (14) suggested as early as 1917 the possibility of C_4 and C_3 units condensing to form a C_7 structure which could cyclize to form a C_6 alicyclic compound with 1 terminal carbon atom. A series of reactions similar to those described by Horecker *et al.* (10) could give rise to a C_7 structure with 3 consecutive carbon atoms derived either from carbon atoms 3 and 4 of

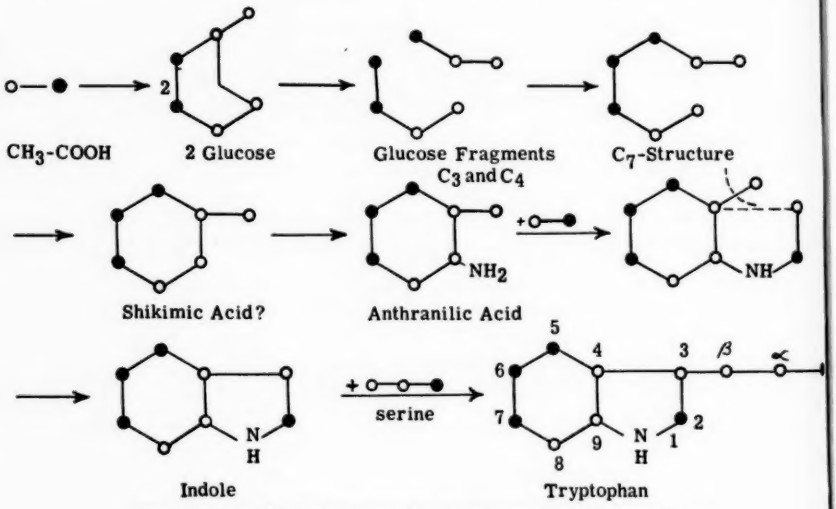


FIG. 1. Hypothetical scheme for the biosynthesis of tryptophan

glucose or the carboxyl carbon of acetate. Cyclization of this between carbons 1 and 6, followed by partial aromatization, would result in the formation of a hydroaromatic ring having the labeling sequence indicated. The relation of this hypothetical C_7 structure to shikimic acid is unknown. Shikimic acid appears to provide the carbon skeleton for anthranilic acid in *Neurospora* (15). Thus, it seems that any scheme proposed to explain the biosynthesis of tryptophan would have to be compatible with the formation of C_7 structures as intermediates.

The mechanism of conversion of anthranilic acid to indole is not known. The data presented in this paper indicate that the carboxyl carbon atom of acetate is a fairly direct source of C_2 of tryptophan. Indole may be formed by acetylation of the amino group of anthranilic acid (or most probably of

some hydroaromatic precursor), followed by elimination of the carboxyl carbon atom and ring closure between the acetate methyl carbon and C_4 of the ring, as shown in Fig. 1. The present experiments do not provide direct evidence for this and are only consonant with previous data showing that the carboxyl carbon atom of anthranilic acid is lost during its conversion to indole (16) and that the amino nitrogen of anthranilic acid is converted to the ring nitrogen of the indole ring (17). Rhuland and Bard (18) demonstrated that the utilization of anthranilic acid by resting cells of *Lactobacillus arabinosus* was stimulated by acetate and coenzyme A. *N*-Acetylanthranilic acid was not used by these cells and appears not to be an intermediate in the conversion of anthranilic acid to indole.

The labeling in the side chain of tryptophan in these experiments was the same as that found in serine and is compatible with the indole-serine condensation mechanism for the formation of tryptophan (19, 20). The results of the degradation of serine and several other amino acids will be the subject of a future communication.

SUMMARY

1. *Aerobacter aerogenes* was cultivated on a medium containing glucose-3,4-C^{14} or acetate-1-C^{14} and unlabeled glucose as the source of carbon. Tryptophan was isolated from the bacterial proteins and subjected to degradation in order to determine the distribution of the isotope.

2. 3 consecutive carbon atoms of the benzene ring and the carboxyl carbon atom of tryptophan originated from carbon atoms 3 and 4 of glucose.

3. The carboxyl carbon atom of acetate, in the presence of unlabeled glucose, gave rise mainly to C_2 of the pyrrole ring of tryptophan.

4. The implications of these and previous results have been discussed.

Addendum—Recent experimental data from this laboratory have indicated that under conditions different from those previously employed the carboxyl carbon atom of acetate is not a precursor for C_2 of tryptophan (*cf.* Table III and Fig. 1). The proposed mechanism for the formation of the pyrrole ring of tryptophan therefore requires revision. The molar ratio of glucose to acetate-1-C^{14} in Experiment II (Table III) was 0.67. If this ratio is increased to 4.0, the per cent of total labeling in tryptophan present in C_2 is reduced from 64 to 10 per cent. A further increase of the molar ratio to 10 results in no incorporation of the acetate carboxyl carbon into C_2. Additional experiments with uniformly labeled C^{14}-glucose have revealed that neither acetate nor pyruvate effectively reduces the labeling in C_2 and C_3, whereas hydroxypyruvate and ribose did effectively reduce the specific activity of these two carbon atoms. The details of these experiments will be the subject of a future communication.

BIBLIOGRAPHY

1. Rafelson, M. E., Ehrensvärd, G., Bashford, M., Saluste, E., and Hedén, C.-G., *J. Biol. Chem.*, **211**, 725 (1954).

2. Rafelson, M. E., *J. Biol. Chem.*, **212,** 953 (1955).
3. Cutinelli, C., Ehrensvärd, G., Reio, L., Saluste, E., and Stjernholm, R., *Acta chem. Scand.*, **5,** 353 (1951).
4. Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159,** 475 (1945).
5. Gilvarg, C., and Bloch, K., *J. Biol. Chem.*, **193,** 339 (1951).
6. Ehrensvärd, G., and Reio, L., *Ark. Kemi*, **5,** No. 20, 229 (1953).
7. Horecker, B. L., and Smyrniotis, P. Z., *J. Am. Chem. Soc.*, **74,** 2123 (1952).
8. Horecker, B. L., and Smyrniotis, P. Z., *J. Cell. and Comp. Physiol.*, **38,** suppl. 1, 137 (1953).
9. Horecker, B. L., Smyrniotis, P., and Klenow, H., *J. Biol. Chem.*, **205,** 661 (1953).
10. Horecker, B. L., Gibbs, M., Klenow, H., and Smyrniotis, P. Z., *J. Biol. Chem.*, **207,** 393 (1954).
11. Axelrod, B., Bandursky, R. S., Greiner, C. M., and Jang, R., *J. Biol. Chem.*, **202,** 619 (1953).
12. Gilvarg, C., and Bloch, K., *J. Biol. Chem.*, **199,** 689 (1952).
13. Ehrensvärd, G., 2e Congrès International de Biochimie, Symposium sur le métabolisme microbien, Paris, 72 (1952).
14. Robinson, R., *J. Chem. Soc.*, **111,** 890 (1917).
15. Tatum, E. L., Gross, S. R., Ehrensvärd, G., and Garnjobst, L., *Proc. Nat. Acad. Sc.*, **40,** 271 (1954).
16. Nyc, J. F., Mitchell, H. K., Leifer, E., and Langham, W. H., *J. Biol. Chem.*, **179,** 783 (1949).
17. Partridge, C. W. H., Bonner, D. M., and Yanofsky, C., *J. Biol. Chem.*, **194,** 296 (1952).
18. Rhuland, L. E., and Bard, R. C., *J. Bact.*, **63,** 133 (1952).
19. Tatum, E. L., and Bonner, D., *Proc. Nat. Acad. Sc.*, **30,** 30 (1944).
20. Tatum, E. L., and Shemin, D., *J. Biol. Chem.*, **209,** 671 (1954).

CORTICOTROPINS (ACTH)

III. KINETIC STUDIES ON THE REACTION OF *N*-TERMINAL GROUP REAGENTS WITH α-CORTICOTROPIN, AND *N*-TERMINAL GROUP ANALYSIS

By ANTHONY L. LEVY* and CHOH HAO LI

(From the Hormone Research Laboratory, University of California, Berkeley, California)

(Received for publication, September 21, 1954)

Paper I of this series (1) reported the isolation of α-corticotropin from sheep pituitary glands. In Paper II (2), this hormone was shown to be a polypeptide consisting of thirty-nine amino acids and 2 molecules of ammonia, with a molecular weight of 4541. The present communication is concerned with a study of the *N*-terminal and other free amino groups of α-corticotropin by three different methods; namely, the fluorodinitrobenzene[1] method of Sanger (3), the CS_2 method of Léonis and Levy (4), and the phenyl isothiocyanate method of Edman (5).

EXPERIMENTAL

Reaction with FDNB—A solution of α-corticotropin trichloroacetate (3.88 mg.) in 3.1 ml. of 0.1 M KCl at pH 8.00 and 40° was allowed to react with FDNB (about 0.1 ml.) with vigorous stirring in order to keep the solution saturated with the reagent; the pH was maintained at 8.00 by means of small additions of standard alkali (0.10 M) from a Jacobsen-Léonis autotitrator (6). The reaction was complete after 1.5 hours, as can be seen from the flattening of the alkali uptake curve (Fig. 2) to the constant end-slope due to the hydrolysis of FDNB (0.016 μmole per $ml.^{-1}$ $min.^{-1}$); under these conditions, the DNP-α-corticotropin remained soluble. Excess FDNB was extracted with ether, and the derivative,[2] after being precipitated by acidification and washed with ether and water, was hydrolyzed for 24 hours at 110° with 0.5 ml. of constant boiling hydrochloric acid in a sealed evacuated tube. Extraction of the diluted hydrolysate with ether, followed by quantitative two-dimensional chromatography of the extract in the "toluene"-1.5 M phosphate system (7), showed DNP-serine to be the sole terminal group, but in an amount equivalent to only 0.1

* Deceased, August 22, 1954.

[1] The following abbreviations are used in this paper: FDNB = 1,2,4-fluorodinitrobenzene; DNP = 2,4-dinitrophenyl; ϕNCS = phenyl isothiocyanate.

[2] Yield of dried material, 4.08 mg., which represents 104 per cent of theory, assuming that 8 DNP residues per mole have been introduced.

mole per mole (4541 gm.) of α-corticotropin. Dinitrophenol was also present to the extent of 0.5 mole per mole, together with traces (about 0.02 mole per mole) of a yellow spot running to the position of bis-DNP-histidine. The small quantity of DNP-serine which was obtained could be explained either in terms of an impurity or as due to an unusual condition of the *N*-terminal residue of α-corticotropin, which might prevent complete reaction with FDNB or give rise to unprecedented destruction during hydrolysis.

That one other amino group (presumably *N*-terminal), in addition to the four ε-NH_2 groups of lysine, had in fact been labeled with a DNP residue is suggested by the following two considerations. (*a*) The ultraviolet ab-

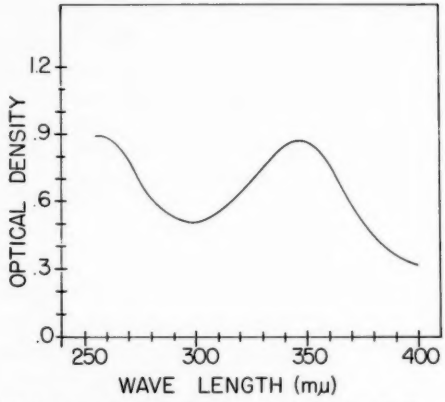


FIG. 1. Ultraviolet absorption of DNP-α-corticotropin (3.26 mg. in 50 ml. of 97 per cent acetic acid solution). Cell width, 1.00 cm.

sorption (Fig. 1) of DNP-α-corticotropin (3.26 mg.) at 350 mμ in 97 per cent acetic acid solution (50 ml.) indicated that substitution by 5.0 N-DNP residues per mole had occurred, if one uses the value of 14,500 which Battersby and Craig (8) have found for the molecular extinction coefficient of the N-DNP residue in polypeptides, and if a correction is made for the small absorption (15 per cent) due to O-DNP-tyrosine and Im-DNP-histidine at this wave-length.[3] Thus, the corrected optical density due to N-DNP groups at 350 mμ = 0.870 × (5/5.45) = 0.798 (assuming five N-DNP, two O-DNP, and one Im-DNP groups per mole); hence the

[3] N-DNP refers to substitution by a dinitrophenyl residue at the amino (NH_2) group of the amino acid, O-DNP refers to substitution by a dinitrophenyl residue at the phenolic hydroxyl (—OH) group of the amino acid (tyrosine), and Im-DNP to substitution by the dinitrophenyl residue at a nitrogen in the iminazole ring of the amino acid (histidine).

micromoles of N-DNP groups = (0.798 × 50)/14.5 = 2.75. But, the micromoles of DNP-α-corticotropin = 3.26/5.87 = 0.55 (assuming eight DNP groups per mole; mol. wt., 5871); hence the moles of N-DNP groups per mole of DNP-α-corticotropin = 2.75/0.55 = 5.0. (*b*) Inspection of the curve of alkali uptake (Fig. 2) resulting from reaction of the hormone with a constant concentration of FDNB at pH 8.00 and 40° indicated that 13.0 equivalents of hydroxide ion were consumed per mole of α-corticotropin, after correction had been made for the alkali uptake resulting from hydrolysis of the reagent. Kinetic analysis (Fig. 3) of the resulting curve showed that of these 13 equivalents 11.2 were utilized in a rapid first order process (half life = 3 minutes) and 1.8 in a slow first order process (half life = 23 minutes). The latter may perhaps be attributed to the reaction of the 2 tyrosine residues, leaving the former to result from the reaction of five amino groups, each liberating 2 protons per mole, and 1 histidine residue (uncharged at pH 8) liberating 1 proton per mole. In a duplicate experiment, 11.1 equivalents of hydroxide ion were consumed during the fast reaction and 3.0 equivalents during the slow.

Further evidence for the fact that there had been complete substitution of the reactive groups of α-corticotropin with DNP residues and confirmation of the identity of the *N*-terminal amino group as serine were provided by examination of the aqueous portion which remained after extraction with ether of the hydrolysate of the DNP hormone. Thus, by direct two-dimensional paper chromatography in the system (9) butanol-acetic acid/*m*-cresol-phenol (1:1), saturated with a borate buffer of pH 9.3, lysine, tyrosine, and histidine were found to be present, having been completely converted to their mono-DNP derivatives. By retreatment of this solution with FDNB (for 80 minutes at pH 9.00 and 40°) followed by quantitative two-dimensional paper chromatography of the resulting DNP derivatives (7), all those residues which had reacted with FDNB were reduced in amount (Table I), serine in particular having diminished from 3 to 2 residues per mole. Partial loss of tyrosine and lysine can doubtless be ascribed to decomposition of their ω-substituted DNP derivatives during hydrolysis. In the case of lysine, incomplete liberation from peptide linkage also appears to be involved, for under somewhat milder conditions of hydrolysis the content of free ε-DNP-lysine fell sharply, and peptides of ε-DNP-lysine appeared. From the alkali uptake accompanying the reaction of the aqueous solution with FDNB, it could, furthermore, be calculated (7) that the hydrolysate was derived from 0.69 μmole of DNP-α-corticotropin. This value was in good agreement with the actual amount (0.67 μmole, assuming eight DNP groups per mole) employed.

Hydrolysis of DNP-serine was studied alone and in the presence of corticotropin and DNP-corticotropin. Two experiments were carried out under somewhat different conditions. The first was on a scale of 1 μmole, hydrol-

ysis being performed for 7 hours at 105° in a sealed evacuated tube with constant boiling HCl in the presence of DNP-corticotropin. The second was performed in duplicate on a scale of 0.2 μmole, and the DNP-α-corticotropin was prepared *in situ* by Sanger's method (3); *i.e.*, 2 hours shaking at room temperature with excess FDNB in 66 per cent ethanol containing sodium bicarbonate. Hydrolysis in this case was performed for 16 hours with constant boiling hydrochloric acid at 110°. In both experiments analyses for recovered DNP-serine were made by quantitative two-dimen-

TABLE I

Molar Ratios in Hydrolysates of α-Corticotropin before and after Dinitrophenylation*

Amino acid	DNP-α-corticotropin		α-Corticotropin†
	Sample 1	Sample 2	
Aspartic acid	2.1	2.0	2.0
Glutamic "	4.9	4.8	4.8
Serine‡	1.8	1.6	2.6
Glycine	3.0	3.1	3.0
Alanine	3.1	3.0	2.9
Proline	4.2	4.3	4.0
Valine	2.8	3.2	3.0
Methionine	(0.1)§	0.9	0.8
Leucine	1.0	1.1	1.0
Phenylalanine	3.0	2.9	3.0
Tyrosine	1.5	1.6	2.1
Lysine	3.0	3.1	3.9
Histidine	0.8	0.9	1.0
Arginine	2.8	(1.6)§	3.0

* 24 hour hydrolysates, 110°, 5.7 N HCl.

† Taken from Levy *et al.* (2).

‡ Uncorrected.

§ Low owing to excessive destruction during hydrolysis.

sional paper chromatography (7); the percentage of recovery from the chromatograms of unhydrolyzed DNP-serine controls was in all cases close to the standard value of 93. The results of these experiments are summarized in Table II. It will be seen that the inherent destruction of DNP-serine (about 25 per cent) is increased somewhat in the presence of corticotropin, but is actually diminished in the presence of DNP-corticotropin. A similar state of affairs was noted by Desnuelle *et al.* (10) in the case of the *N*-terminal DNP-aspartic acid of serum albumin. In all cases, the yield of *N*-terminal DNP-serine from DNP-corticotropin alone was no greater than 0.1 to 0.2 mole per mole, a value which is, of course, little affected by the above correction for hydrolysis.

Examination of the ether washings from the preparation of DNP-α-corticotropin, after sublimation (11) of excess dinitrophenol, showed that DNP-serine was not lost prematurely, as is the case with the terminal residues of "old" yellow enzyme (12) and carboxypeptidase (13). The yield of DNP-serine could not be increased beyond 0.1 to 0.2 mole per mole of α-corticotropin, either by decreasing the time of hydrolysis, by hydrolyzing DNP-α-corticotropin with concentrated hydrochloric acid at 40° for 25 days, or by extending the original period of reaction with FDNB.

Reaction with CS_2—Reaction of CS_2 with α-corticotropin trichloroacetate (5 mg.) in 3.1 ml. of 0.1 M KCl at a constant pH of 8.00 and constant concentration of CS_2, and at a temperature of 40°, was a single first

TABLE II

Per Cent of Recovery of Added DNP-Serine Following Hydrolysis in Presence of α-Corticotropin and Its DNP Derivative

Experiment No.*	Amount of constituent subjected to hydrolysis			Recovery of DNP-serine
	DNP-serine	α-Corticotropin	DPN-α-corticotropin	
	μM	μM	μM	*per cent*
1	1.0	0	0	86
1	1.0	0	1.0	93
2	0.2	0	0	69, 67
2	0.2	0.2	0	52, 47
2	0.2	0	0.2	70, 78

* Experiment 1, hydrolysis carried out for 7 hours at 105° with 5.7 N HCl; Experiment 2, hydrolysis carried out for 16 hours at 110° with 5.7 N HCl.

order process (Figs. 2 and 3), but was 35 times slower than the corresponding reaction with FDNB. In the present case, however, it is known (14) that only free NH_2 groups react; hence the fact that 10.0 equivalents of hydroxide ion were consumed per mole of α-corticotropin again points to the presence of one NH_2 group in addition to the four ε-NH_2 groups of lysine.

Acidification of the clear solution of α-corticotropin dithiocarbamate caused precipitation of the derivative, but extraction with ether did not yield any 2-thio-5-thiazolidone. This behavior is in accord with the relative inability of CS_2 to detach the terminal residues of polypeptides and proteins, as distinct from oligopeptides, and is discussed in detail elsewhere (4).

Reaction with Phenyl Isothiocyanate—The Edman degradation was carried out according to the modifications proposed by Ottesen and Wollenberger (15) and Fraenkel-Conrat (16). Because of the slowness of the re-

action, coupling of α-corticotropin trichloroacetate (5 mg.) with a suspension of φNCS in 3.1 ml. of 0.1 M KCl solution at pH 8.0 and at 40° was carried out in a 50 per cent dioxane solution, in which the reagent was more soluble; the course of the reaction was followed in the usual way by recording (Fig. 2) the additions of standard alkali needed to maintain a constant pH. From the logarithmic plot (Fig. 3) after subtraction of the end-slope, it may be seen that the reaction follows approximately first

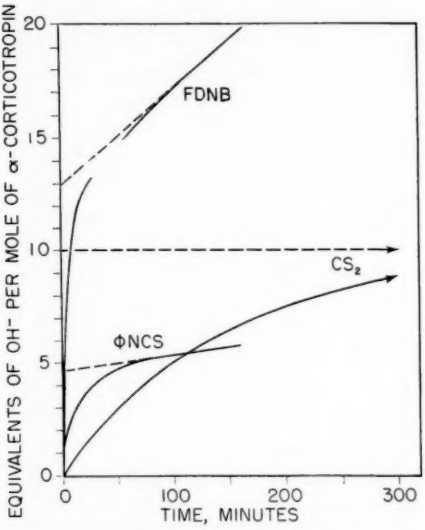


FIG. 2. Alkali uptake as a function of time for the reaction of α-corticotropin with fluorodinitrobenzene (FDNB), CS_2, and phenyl isothiocyanate (φNCS) at 40°, pH 8.00, and 0.1 ionic strength. Solvents, water, FDNB, and CS_2; 50 per cent dioxane, φNCS. The broken line in each case indicates the terminal slope of the curve.

order kinetics. Unlike the coupling of FDNB and CS_2, the reaction of φNCS with amino groups does not result in the formation of new acidic centers, and hence the pH change accompanying the reaction is due entirely to the dissociation of hydrogen ions from $—NH_3^+$ groups, which occurs as the latter are converted to neutral phenylthiocarbamyl derivatives. Hence, the alkali representing the change in titer resulting from the disappearance of groups which dissociate in the titration range of pH 5.6 to 8.0 (0.4 equivalent per mole of α-corticotropin) was added to the alkali consumed during the first order reaction (4.7 equivalents per mole of α-corticotropin); these values together indicate once again the presence of five reactive amino groups in the α-corticotropin molecule. This correc-

tion (15) was not attempted in the case of reactions with FDNB and CS_2, partly because it is less significant in these cases and partly because interference could be expected from the dinitrophenol and the dithiocarbamic acid which are formed during the reaction.

After extraction of the excess ϕNCS and of material absorbing light at 270 mμ, an equal volume of 5 N hydrochloric acid was added and the solution extracted with ethyl acetate after having stood for 2 hours at room temperature. Light absorption of the extract ($\lambda_{max.}$ = 270 mμ) indicated the presence of about 0.6 mole of phenylthiohydantoin per mole of α-cor-

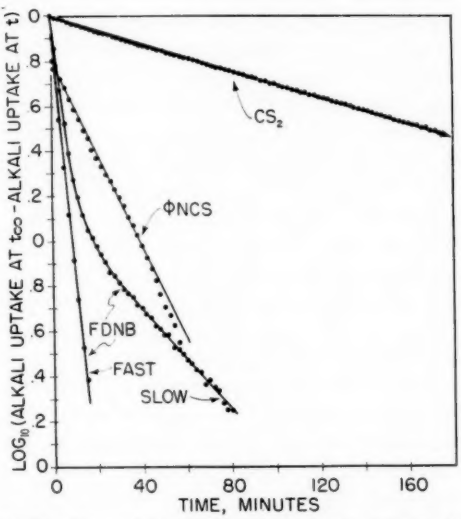


FIG. 3. Kinetic analysis of the alkali uptake curves of Fig. 2

ticotropin, which was identified as the serine derivative from the results of paper chromatography in the systems of Sjöquist (17) and Landmann *et al.* (18), and from the fact that, when it is heated with diluted acid (30 minutes, 100°, 0.1 N HCl), its light absorption maximum is at 320 mμ; this behavior is characteristic of serine in the absence of threonine and cystine.[4] However, from the shape of the absorption curve and from the fact that a sizable origin spot was observed on the paper chromatograms, it appears that the serine phenylthiohydantoin was partly polymerized. For this reason, the quantitative aspect can be regarded as only approximate, since the molar extinction coefficient of the polymer is not known.

By employing the paper strip technique of Fraenkel-Conrat (19) for ef-

[4] A. L. Levy, unpublished results.

fecting the Edman degradation, Harris and Li (20) have raised the yield of serine phenylthiohydantoin to 0.85 mole per mole and have obtained a derivative virtually free of polymer.

DISCUSSION

The kinetic approach to the study of the reaction with α-corticotropin of reagents used for the analysis of *N*-terminal groups is based upon the work of Léonis and others (14, 4, 15). According to this procedure, a constant concentration of the reagent is maintained by using a saturated solution, and the kinetics are followed by noting the rate of addition of hydroxide ions necessary to maintain the pH of the solution at a constant value. Under these conditions, the kinetics of the reaction of the reagent with amino groups are reduced from second to first order, and the kinetics of its concomitant hydrolysis by hydroxide ions are reduced from second to zero order. It is then a simple matter to isolate the alkali uptake resulting from the reaction with the amino groups by subtraction of the linear end-slope (Fig. 2). Hydrolysis was marked in the case of FDNB, was less with φNCS, and was inappreciable in the case of CS_2. When the log (alkali uptake at t_∞ less alkali uptake at t) for the reaction of the amino groups was plotted against time (Fig. 3), straight lines were obtained for the reactions with φNCS and CS_2, confirming the first order character of these condensations. In the case of the FDNB reaction a straight line was obtained after about 20 minutes, when the initial rapid reaction was over. From the slope of this line and the extrapolated value at $t = 0$, the alkali uptake curve for the slow reaction could be reconstructed. Subtraction of this from the curve for the over-all reaction isolated the fast reaction, which also turned out to be a single first order process, affording a straight line log plot (Fig. 3). The first order velocity constants for the reaction of α-corticotropin with FDNB, CS_2, and φNCS in solutions saturated with the reagent at 40° are summarized in Table III.

Although only few data relating to the kinetics of acylation of polypeptides and proteins are at present available, it is apparent that α-corticotropin behaves in a somewhat exceptional fashion. Thus, the rate of condensation of its amino groups with FDNB is about 20 times more rapid than that of free lysine at the same pH, and about 4 times more rapid than that of the free amino groups of proteins such as insulin and growth hormone.[5] Also, the fact that the terminal amino group liberates 2 protons at pH 8 suggests that its pK is closer to that of the ε-NH_2 groups of lysine (pK 10.1) than to the normal value for α-NH_2 groups (about pK 7.5). This unusual behavior is paralleled in the condensation of α-corticotropin

[5] A. L. Levy and C. H. Li, to be published.

and CS_2. Here again the equivalence of the five amino groups is indicated by the liberation of 10 protons per mole, and, further, by the occurrence of a straightforward first order reaction, in contrast with what is usually the case; namely, that α-amino groups react more rapidly than ϵ-amino groups at pH 8.0 and can be revealed by kinetic analysis (14). The rate of reaction of CS_2 with the amnio groups of α-corticotropin is about 6 times more rapid than with either the ϵ-amino groups of lysine itself (14) or with the ϵ-amino groups of the several proteins so far studied.

The anomalous character of α-corticotropin is exemplified most strongly by the fact that its *N*-terminal DNP-serine is destroyed to the extent of 80 to 90 per cent during hydrolytic cleavage from the DNP hormone, and that this destruction cannot be corrected for by hydrolysis of a control

TABLE III

First Order Rate Constants for Reactions of α-Corticotropin with N-Terminal End-Group Reagents*

Reagent	Solvent†	k (fast)	k (slow)
FDNB	Water	0.24	0.026
"	"	0.23	0.034
CS_2	"	0.0067	
ϕNCS	50% dioxane	0.047	

* Log_e min.$^{-1}$.

† Containing KCl (0.1 M) in all cases.

sample of DNP-serine in the presence of α-corticotropin or DNP-α-corticotropin. In this respect α-corticotropin is unique in the literature of the DNP method (21). This investigation makes clear the need for caution in interpreting the occurrence of submolar quantities of an end-group as revealed by the DNP method, and emphasizes the advisability of an approach to the problem which makes use of the various methods available.

The analysis of the hydrolysate of DNP-α-corticotropin demonstrates how an end-group determination and an amino acid analysis can be made on the same micro sample of peptide. The results, however, indicate that less reliable values are obtained for those amino acids (lysine, tryosine, as well as the *N*-terminal residue) which react with FDNB, and hence that quantitative amino acid analyses should be made on hydrolysates of untreated samples whenever possible.

N-Terminal serine has also been found by Landmann, Drake, and White (22) in corticotropin A, derived from pig pituitaries. These investigators employed the methods of both Sanger and Edman; in neither

XUM

case were quantitative data reported. However, Fox *et al.* (23) reported on *N*-terminal serine in corticotropin A, determined by means of a subtractive method employing the phenylthiohydantoin derivative; in essence, this procedure is similar to that recorded in Table I.

SUMMARY

The kinetics of reaction of the *N*-terminal group reagents FDNB, CS_2, and ϕNCS with α-corticotropin at constant pH 8.0 have been studied. In all cases, the amount of alkali consumed by the reaction pointed to one free amino group (per 4541 mol. wt.) in addition to the reactive side chains of the amino acids of the peptide. That this free amino group belonged to *N*-terminal serine was shown by the isolation of DNP-serine and serine phenylthiohydantoin after treatment with acid. DNP-serine was obtained in this manner in only 10 to 20 per cent of the theoretical yield, which could not be corrected for along conventional lines. However, amino acid analysis of α-corticotropin after treatment with FDNB revealed that 1 whole serine residue had disappeared. The anomalous character of α-corticotropin with respect to kinetic behavior is discussed.

We wish to acknowledge research grants from the National Institutes of Health, United States Public Health Service (No. G-2907), and from Eli Lilly and Company, which have greatly aided this investigation. Special thanks are due to Mr. David Chung for his expert technical assistance.

BIBLIOGRAPHY

1. Li, C. H., Geschwind, I. I., Dixon, J. S., Levy, A. L., and Harris, J. I., *J. Biol. Chem.*, **213**, 171 (1955).
2. Levy, A. L., Geschwind, I. I., and Li, C. H., *J. Biol. Chem.*, **213**, 187 (1955).
3. Sanger, F., *Biochem. J.*, **39**, 507 (1945).
4. Léonis, J., and Levy, A. L., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **29**, 57 (1954).
5. Edman, P., *Acta chem. Scand.*, **4**, 283 (1950).
6. Jacobsen, C. F., and Léonis, J., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **27**, 333 (1951).
7. Levy, A. L., *Nature*, **174**, 126 (1954).
8. Battersby, A. R., and Craig, L. C., *J. Am. Chem. Soc.*, **74**, 4023 (1952).
9. Levy, A. L., and Chung, D., *Anal. Chem.*, **25**, 396 (1953).
10. Desnuelle, P., Rovery, M., and Fabre, C., *Compt. rend. Acad.*, **233**, 987 (1951).
11. Mills, G. L., *Biochem. J.*, **50**, 707 (1951–52).
12. Weygand, F., and Junk, R., *Naturwissenschaften*, **38**, 433 (1951).
13. Thompson, E. O. P., *Biochim. et biophys. acta*, **10**, 633 (1953).
14. Léonis, J., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **26**, 315 (1948).
15. Ottesen, M., and Wollenberger, A., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **28**, 463 (1953).
16. Fraenkel-Conrat, H., and Fraenkel-Conrat, J., *Acta chem. Scand.*, **5**, 1409 (1951).

17. Sjöquist, J., *Acta chem. Scand.*, **7**, 447 (1953).
18. Landmann, W. A., Drake, M. P., and Dillaha, J., *J. Am. Chem. Soc.*, **75**, 3638 (1953).
19. Fraenkel-Conrat, H., *J. Am. Chem. Soc.*, **76**, 3606 (1954).
20. Harris, J. I., and Li, C. H., *J. Am. Chem. Soc.*, **76**, 3607 (1954).
21. Levy, A. L., in Glick, D., Methods of biochemical analysis, New York, **2**, in press.
22. Landmann, W. A., Drake, M. P., and White, W. F., *J. Am. Chem. Soc.*, **75**, 4370 (1953).
23. Fox, S. W., Hurst, T. L., and Warner, C., *J. Am. Chem. Soc.*, **76**, 1154 (1954).

CORTICOTROPINS (ACTH)

IV. THE ACTION OF CARBOXYPEPTIDASE ON α-CORTICOTROPIN, AND THE *C*-TERMINAL AMINO ACID SEQUENCE

By J. IEUAN HARRIS and CHOH HAO LI

(From the Hormone Research Laboratory, University of California, Berkeley, California)

(Received for publication, September 21, 1954)

In Paper I of this series (1) the isolation from sheep pituitary glands of a pure polypeptide, α-corticotropin (ACTH), was described. The results of amino acid and terminal group analyses (2–4) indicate that the α-corticotropin molecule is composed of a single polypeptide chain containing thirty-nine amino acid residues. Numerous chemical methods, described in Paper III (4), are available for the identification and quantitative estimation of the *N*-terminal groups of polypeptide chains, but as yet no generally applicable chemical procedures have been developed for a complementary investigation of the corresponding *C*-terminal groups. However, an enzymatic method of *C*-terminal group analysis based on the use of the proteinase carboxypeptidase was proposed by Lens (5), and this method has recently been extended and used with considerable success in many laboratories for the identification and estimation of *C*-terminal groups in polypeptide and protein molecules (6, 7).

The development of a convenient micromethod (8) for the analysis of amino acids as their dinitrophenyl derivatives, which has been used to follow the rate of release of amino acids from a polypeptide chain by the action of carboxypeptidase (9, 10), has facilitated the further application of this enzymatic procedure to the elucidation of amino acid sequences from the carboxyl end. The use of carboxypeptidase for such investigations is based upon the assumption that the enzyme, by analogy with its action on synthetic substrates, attacks specifically only those bonds which are adjacent to free α-COOH groups in polypeptide chains. It follows that the carboxypeptidase method may be applied to determine the sequence of amino acids from the carboxyl end in a protein molecule, provided that the protein substrate does not contain more than *one* open polypeptide chain and that the *C*-terminal peptide bond as well as other points of interaction with the enzyme, such as the adjacent peptide bond and the *C*-terminal amino acid side chain, are accessible and conform to the specificity requirements of the enzyme. With these conditions fulfilled, the enzyme would be expected to release amino acids one by one along a peptide chain, until a structural barrier is encountered as a result

XUM

of failure on the part of a potentially susceptible bond to conform to the specificity requirements necessary for the continued action of the enzyme.

Furthermore, as discussed by Neurath *et al.* (11), since the intrinsic rate of hydrolysis of a peptide bond depends largely on the nature of the side chain of its *C*-terminal amino acid, the rate at which each successive amino acid is released from a polypeptide chain will be directly related to their sequence along the chain if they are arranged in order of decreasing rates of hydrolysis. At any point of inversion of this sequence the rate of hydrolysis of the preceding bond would become the rate-determining step for the hydrolysis of the succeeding one, irrespective of the nature of its side chain; hence the two adjacent amino acids should be released at equal rates. However, in the case of a peptide bond in a long chain polypeptide or protein molecule, this interpretation may not be strictly valid, since its rate of hydrolysis may also be influenced markedly by the over-all environmental conditions imposed on the bond (*e.g.*, steric and electrostatic factors, hydrogen bonding, etc.) by virtue of the size and spatial configuration of the polypeptide chain in a folded molecule.

With the above considerations in mind, the action of carboxypeptidase on α-corticotropin has been studied in an attempt to ascertain the nature of the *C*-terminal residue and to determine the sequence of amino acids from the carboxyl end of the chain in the polypeptide hormone. Preliminary reports of these results have been published (3, 12).

EXPERIMENTAL

α-Corticotropin trichloroacetate was prepared from sheep pituitary glands as described in Paper I (1) and was found to behave as a single substance when examined by zone electrophoresis on starch, partition chromatography, and counter-current distribution.

The enzyme used during the course of this work was a preparation, six times recrystallized, obtained through the courtesy of Dr. W. M. Thompson of Armour and Company. Just before use, the carboxypeptidase crystals were washed with distilled water to remove adsorbed amino acids and brought into solution at pH 8.0 to 8.5 by the dropwise addition with stirring of 1 per cent $NaHCO_3$ at 0–4°. A 50-fold excess of diisopropyl fluorophosphate (DFP) was added to destroy the last traces of endopeptidase activity in the carboxypeptidase preparation (13–15).

Qualitative Studies—When a 1 per cent solution of α-corticotropin (0.2 μmole) was allowed to react with carboxypeptidase (enzyme-substrate mole ratio, 1:100) at pH 8.5 and at a temperature of 25° for 30 minutes, only one amino acid, phenylalanine, could be detected when the whole digest was applied to paper and submitted to chromatography in butanol-acetic acid-H_2O (4:1:5). When the period of digestion was lengthened to 16

hours, however, smaller quantities of two other amino acids, glutamic acid and leucine, also were identified in a similar manner. These amino acids were released much more slowly than the phenylalanine, and on the basis of the assumption that α-corticotropin is composed of only one peptide chain it was tentatively inferred that phenylalanine occurs as its *C*-terminal residue and that glutamic acid and leucine occupy adjacent positions in the chain in an undetermined sequence.

Semiquantitative Estimation of Liberated Amino Acids—In order to obtain a rapid semiquantitative estimate of the amino acids released by the action of carboxypeptidase on α-corticotropin, the "molecular sieve" procedure of Partridge and Thompson (16) was used. α-Corticotropin trichloroacetate (0.2 per cent solution, 0.2 μmole) was incubated with carboxypeptidase (enzyme-substrate mole ratio, 1:80) at 40° for 16 hours at pH 8.5. The addition of 50 mg. of Amberlite IR-120 resin (H form, 20 to 50 mesh, 8 per cent cross-linked) served to terminate the enzyme reaction by lowering the pH of the reaction solution to 3. The suspension containing the resin was shaken mechanically for 1 hour and the supernatant solution removed by means of a pipette; the resin was washed free of residual peptide with distilled water and then with 5 M ammonia (0.5 ml.) to displace the adsorbed amino acids. The ammonia eluate was taken to dryness *in vacuo* over concentrated H_2SO_4, redissolved in distilled water (20 μl.), and examined by two-dimensional chromatography on paper in butanol-acetic acid-H_2O (4:1:5) and 1:1 phenol-*m*-cresol saturated with borate buffer at pH 9.3 (17). As shown in Fig. 1, phenylalanine, glutamic acid, and leucine are present in approximately equimolar proportions, and presumably arise through the stepwise degradation of the α-corticotropin molecule from the COOH end. The yield of the *C*-terminal amino acid phenylalanine was estimated to be approximately 0.15 to 0.20 μmole per 0.2 μmole of corticotropin by comparing its ninhydrin color with that of a graded scale of reference spots of phenylalanine (*cf.* Gladner and Neurath (18)).

Quantitative C-Terminal Group and Sequence Analysis—In order to obtain a quantitative determination of the three amino acids which are released by the action of carboxypeptidase and to ascertain the sequence in which they occur, an additional experiment was designed to follow the rates of their release during the enzymatic reaction.

α-Corticotropin trichloroacetate (0.56 mg. of N) was incubated with carboxypeptidase (0.04 mg. of N; enzyme-substrate mole ratio, 1:100), in a total solution of 5.0 ml. at pH 8.5 and 40°; a constant pH of 8.5 was maintained throughout the course of the enzymatic reaction by the periodic addition of small amounts of 0.05 N KOH from a micro burette.

Aliquots of the reaction solution (1.0 ml. containing 0.112 mg. of α-corticotropin N) were removed after 0.5, 2, 4, 8, and 18 hours and acidified

to pH 3.0 with 0.1 N HCl to terminate the action of the enzyme. Each aliquot was subsequently allowed to react with 0.1 ml. of dinitrofluorobenzene (FDNB) at pH 9.0 and 40° with vigorous stirring in order to maintain saturation with respect to the reagent (8); these reactions at constant pH were carried out in the reaction cell of a Jacobsen-Léonis autotitrator (19). After the reaction had proceeded for 90 minutes, the contents of the reaction vessel were transferred quantitatively to a centrifuge tube (15 ml.), and excess FDNB was extracted by shaking with three 5 ml. portions of peroxide-free ether. The aqueous layer was then

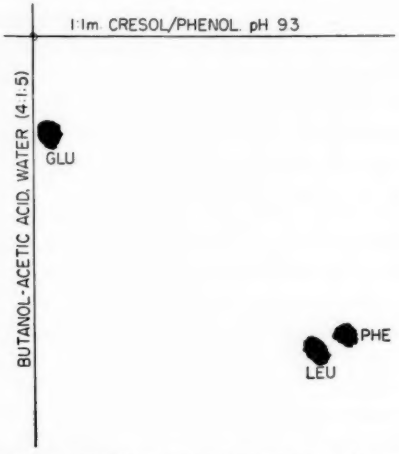


FIG. 1. Amino acids released from α-corticotropin by the action of carboxypeptidase.

adjusted to pH 1 with 1 N HCl, and the 2,4-dinitrophenyl (DNP) derivatives of those amino acids liberated during the carboxypeptidase reaction were separated from the DNP derivative of residual α-corticotropin by means of thorough extraction of the acidified suspension with 5 ml. portions of peroxide-free ether. The DNP peptide was collected by centrifugation, and the combined ether extracts were taken to dryness *in vacuo*. Dinitrophenol (formed by hydrolysis of FDNB at pH 9.0) was removed by sublimation *in vacuo* at 60° onto a cold finger containing solid CO_2 (20). The residual DNP amino acids were analyzed by two-dimensional chromatography on paper in the "toluene"-1.6 M phosphate buffer system (8). A typical chromatogram (derived from the 18 hour aliquot) is shown in Fig. 2; as expected, spots were obtained corresponding to the DNP derivatives of phenylalanine, glutamic acid, and leucine. Each DNP amino acid was

eluted separately from each chromatogram with 1 per cent $NaHCO_3$ (4 ml.), and its quantity estimated from the ultraviolet absorption at 360 mμ.

In order to ascertain the extent of recovery in the presence of DNP-α-corticotropin of phenylalanine, glutamic acid, and leucine as their DNP derivatives, a control experiment was designed to simulate the conditions employed during the procedure used for the release and subsequent estimation of the amino acids formed during the reaction between α-corticotropin

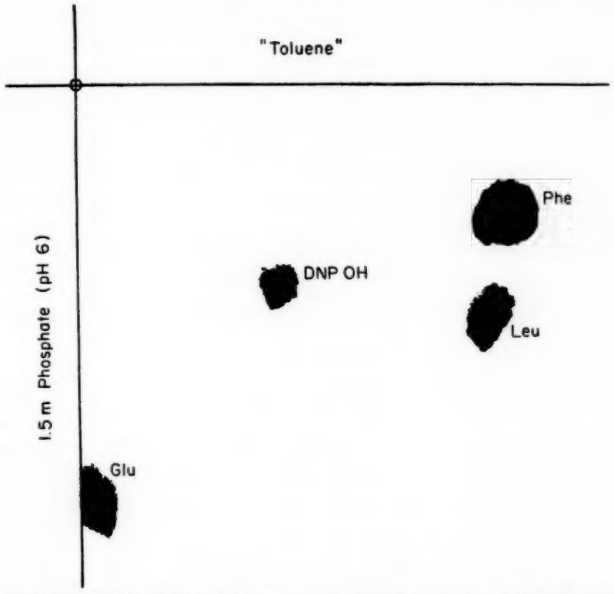


FIG. 2. Amino acids released from α-corticotropin by the action of carboxypeptidase, identified as their DNP derivatives.

and carboxypeptidase. α-Corticotropin trichloroacetate (0.5 mg. of N) was dissolved in water (5 ml.) containing 1 μmole each of phenylalanine, glutamic acid, and leucine. The solution was incubated at pH 8.5 and 40° for 90 minutes. After removal of excess reagent, the solution was acidified to pH 1.0 and the DNP amino acids were recovered by extraction; triplicate aliquots of the ether extract (corresponding to one-tenth of the total extract) were submitted to two-dimensional chromatography on paper, eluted with 1 per cent $NaHCO_3$, and their optical densities determined at 360 mμ as described above. The results obtained, together with the calculated effective millimolar extinction coefficients (8) for the three DNP amino acids, are recorded in Table I. It is considered that the experi-

mental losses (*inter alia*, losses due to incomplete extraction of the DNP amino acids in the presence of DNP peptide, losses on paper during chromatography, etc.) are reflected in the calculated "effective" molecular

TABLE I

Effective Millimolar Extinction Coefficients of DNP-Phenylalanine, DNP-Glutamic Acid, and DNP-Leucine Prepared in Presence of α-Corticotropin and Subsequently Chromatographed on Paper

DNP amino acid	Optical density at 360 mμ				Effective mM extinction coefficient*
	Sample 1†	Sample 2†	Sample 3†	Mean	
Phenylalanine	0.354	0.348	0.359	0.354	14.2 (15.2)‡
Glutamic acid	0.402	0.421	0.420	0.414	16.6 (16.6)
Leucine	0.357	0.362	0.361	0.360	14.4 (14.2)

* As defined by Levy (8).

† 0.1 μmole aliquot.

‡ The values in parentheses are standard values taken from Levy (8).

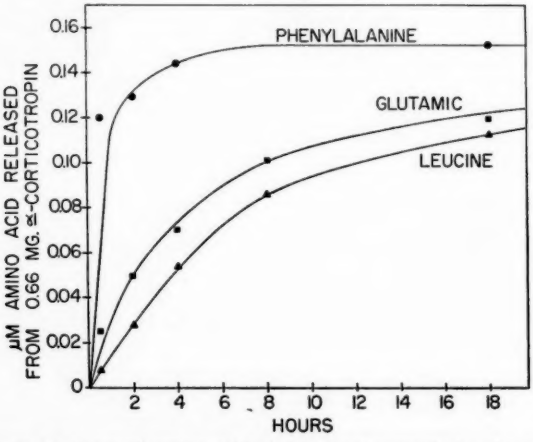


FIG. 3. Rate of release of amino acids from α-corticotropin by the action of carboxypeptidase, estimated as their DNP derivatives.

extinction coefficients; consequently, these latter values have been used for calculating the yields, in micromoles of the amino acids liberated in the carboxypeptidase reaction, from corresponding optical density readings.

Results showing the rates of release of phenylalanine, glutamic acid, and leucine from α-corticotropin are presented in Fig. 3. The *C*-terminal amino acid, phenylalanine, is completely released under the conditions of

the experiment and attains a plateau value corresponding to 0.153 μmole (±0.006) from 0.66 mg. of α-corticotropin. If it is assumed that 1 mole of phenylalanine is released per mole of α-corticotropin, a value of 4300 (±200) may be calculated for its equivalent weight; this value is in good agreement with the minimal molecular weight of α-corticotropin (4541) calculated on the basis of its amino acid composition (2).

Glutamic acid and leucine are released successively in this sequence but at much slower rates, and attain values equivalent to approximately 75 and 65 per cent, respectively, of the phenylalanine only after 18 hours of digestion.

However, when α-corticotropin which had been digested for 18 hours under the conditions described above was precipitated at pH 5.5 to 6.0 and again treated with fresh enzyme, additional amounts of glutamic acid and leucine, estimated as 0.2 to 0.3 mole of each amino acid by spot dilution chromatography (*cf.* (18)), were released, whereas phenylalanine, according to expectation, was now completely absent. No other amino acids were released, even after prolonged digestion[1] with high concentrations of enzyme, an observation which would seem to indicate a structural barrier to further degradation along the peptide chain.

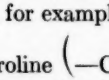
DISCUSSION

From the results of the present investigation it is concluded that phenylalanine, glutamic acid, and leucine are released successively in equivalent stoichiometric proportions when α-corticotropin is allowed to react with carboxypeptidase, and that they arise through the stepwise degradation of the *C*-terminal sequence . . . Leu.Glu.Phe in the peptide hormone.

The nature of the enzymatic reaction is consistent with what is known about the specificity characteristics of carboxypeptidase. As expected, the *C*-terminal phenylalanine is released rapidly despite the presence of a γ-COOH group in the side chain of the adjacent residue, glutamic acid. After the release of phenylalanine, however, glutamic acid is exposed as the new *C*-terminal residue; a charged group in the side chain of a *C*-terminal residue is known to retard the action of the enzyme (24, 25), and consequently the slow rate of release of glutamic acid was expected. In addition, the rate of release of glutamic acid should determine the rate at which the peptide bond involving the COOH group of the next amino acid along

[1] α-Corticotropin from which the three *C*-terminal amino acids (Phe, Glu, and Leu) had been quantitatively removed by the action of carboxypeptidase (after two cycles of reaction, as described above) was found to retain hormonal activity. It is significant that insulin (21), lysozyme (J. I. Harris, unpublished data), tobacco mosaic virus (22), growth hormone (10), and α-chymotrypsin (23) have also been found to retain their respective biological functions after the liberation of their respective *C*-terminal amino acid residues.

the chain, leucine, becomes available to the enzyme. On the basis of side chain affinity, leucine with its fatty side chain should be released as rapidly as it becomes available to the enzyme, theoretically at a rate equal to that of the preceding residue, glutamic acid. However, leucine is released at a rate which is significantly slower than that of glutamic acid.

The fact that no further reaction takes place after stoichiometric amounts of phenylalanine, glutamic acid, and leucine have been released in the course of the reaction with carboxypeptidase suggests that the fourth amino acid along the chain is one which does not conform to the specificity requirements of the enzyme and consequently resists digestion. Studies on synthetic substrates have shown that peptide bonds involving *C*-terminal proline are for all practical purposes completely resistant to the action of carboxypeptidase (25). Furthermore, in order to exert its maximal effect, carboxypeptidase requires the presence of a normal —CO—NH— peptide bond in a position adjacent to the susceptible *C*-terminal linkage. The presence, for example, of an adjacent peptide bond involving the ring nitrogen of proline  is known to retard, and in some cases to inhibit completely, the release of the *C*-terminal amino acid (24, 25).

In the light of these findings, the occurrence of the *C*-terminal tetrapeptide sequence ...Pro.Leu.Glu.Phe in the α-corticotropin molecule could account for the apparent structural barrier to further enzymatic digestion after the quantitative liberation of the *C*-terminal amino acids (phenylalanine, glutamic acid, and leucine) and for the unusually slow release of leucine when it in turn becomes the *C*-terminal amino acid after the removal of phenylalanine and glutamic acid.

Evidence which indicates that the above *C*-terminal tetrapeptide sequence does in fact occur in α-corticotropin was provided by the isolation and characterization from a pepsin digest of the hormone, the tetrapeptide Pro.Leu.Glu.Phe (3). The R_F value of this fragment was 0.83 when it was submitted to chromatography on Whatman No. 1 paper in butanol-acetic acid-H_2O, 4:1:5 (see White (26)). Since it is known that only 1 residue of leucine is present in the molecule (2), the tetrapeptide Pro.Leu.Glu.Phe is shown to be the *C*-terminal sequence in α-corticotropin. It is worthy of note that corticotropin A, isolated from hog pituitary glands (27), has been reported to have the same *C*-terminal tetrapeptide sequence (26).

SUMMARY

When α-corticotropin is allowed to react with carboxypeptidase, phenylalanine, glutamic acid, and leucine are shown to be released successively and in equivalent stoichiometric amounts. From theoretical considerations of the kinetic data and the isolation of the tetrapeptide Pro.Leu.

Glu.Phe from a pepsin digest, it is concluded that the amino acids are released by the stepwise degradation of the *C*-terminal tetrapeptide sequence ...Pro.Leu.Glu.Phe in the peptide hormone.

The authors wish to acknowledge the able assistance of Ning G. Pon during the course of this investigation. It is a pleasure to record our appreciation of research grants from the National Institutes of Health, United States Public Health Service (No. G-2907), and from Eli Lilly and Company.

BIBLIOGRAPHY

1. Li, C. H., Geschwind, I. I., Dixon, J. S., Levy, A. L., and Harris, J. I., *J. Biol. Chem.*, **213,** 171 (1955).
2. Levy, A. L., Geschwind, I. I., and Li, C. H., *J. Biol. Chem.*, **213,** 187 (1955).
3. Harris, J. I., and Li, C. H., *J. Am. Chem. Soc.*, **76,** 3607 (1954).
4. Levy, A. L., and Li, C. H., *J. Biol. Chem.*, **213,** 487 (1955).
5. Lens, J., *Biochim. et biophys. acta*, **3,** 367 (1949).
6. Jutisz, M., *Bull. Soc. chim. biol.*, **36,** 109 (1954).
7. Harris, J. I., in Glick, D., Methods of biochemical analysis, New York, **2,** in press.
8. Levy, A. L., *Nature*, **174,** 126 (1954).
9. Anfinsen, C. B., Redfield, R. R., Choate, W. L., Page, J., and Carroll, W. R., *J. Biol. Chem.*, **207,** 201 (1954).
10. Harris, J. I., Li, C. H., Condliffe, P. G., and Pon, N. G., *J. Biol. Chem.*, **209,** 133 (1954).
11. Neurath, H., Gladner, J. A., and Davie, E. W., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, 50 (1954).
12. Li, C. H., Geschwind, I. I., Levy, A. L., Harris, J. I., Dixon, J. S., Pon, N. G., and Porath, J. O., *Nature*, **173,** 251 (1954).
13. Jansen, E. F., Nutting, M.-D. F., Jang, R., and Balls, A. K., *J. Biol. Chem.*, **179,** 189 (1949).
14. Gladner, J. A., and Neurath, H., *Biochim. et biophys. acta*, **9,** 335 (1952).
15. Sanger, F., and Thompson, E. O. P., *Biochem. J.*, **53,** 366 (1953).
16. Partridge, S. M., *Nature*, **169,** 493 (1952). Thompson, A. R., *Nature*, **169,** 495 (1952).
17. Levy, A. L., and Chung, D., *Anal. Chem.*, **25,** 396 (1953).
18. Gladner, J. A., and Neurath, H., *J. Biol. Chem.*, **205,** 345 (1953).
19. Jacobsen, C. F., and Léonis, J., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **27,** 333 (1951).
20. Mills, G. L., *Biochem. J.*, **50,** 707 (1951–52).
21. Harris, J. I., and Li, C. H., *J. Am. Chem. Soc.*, **74,** 2945 (1952).
22. Harris, J. I., and Knight, C. A., *Nature*, **170,** 613 (1952).
23. Gladner, J. A., and Neurath, H., *J. Biol. Chem.*, **206,** 911 (1954).
24. Neurath, H., and Schwert, G. W., *Chem. Rev.*, **46,** 69 (1950).
25. Smith, E. L., in Wolstenholme, G. E. W., and Cameron, M. P., Chemical structure of proteins, Ciba Foundation symposium, Boston, 109 (1954).
26. White, W. F., *J. Am. Chem. Soc.*, **75,** 4877 (1953).
27. White, W. F., *J. Am. Chem. Soc.*, **75,** 503 (1953).

THE UTILIZATION OF NUCLEOTIDES BY THE MAMMAL

II. N^{15}-LABELED PURINE NUCLEOTIDES*

By PAUL M. ROLL† AND IRVING WELIKY

(From the Laboratories of the Sloan-Kettering Division of Cornell University Medical College, New York, New York)

(Received for publication, September 28, 1954)

Although mammals have no dietary requirement for any of the organic constituents derived from nucleic acids, it seemed likely that such compounds might well be intermediates in the biosynthesis of nucleic acids. With the advent of isotopic tracer techniques it was possible to demonstrate that certain nucleic acid components, when made available to animals, were actually incorporated into tissue polynucleotides. Thus, adenine was shown to be an excellent precursor of the adenine and a somewhat poorer precursor of the guanine of the PNA[1] in the rat (3). A quite small incorporation of guanine into PNA guanine was also observed (4). No incorporation of the pyrimidines cytosine (5), uracil, or thymine (6) was detected. These results, and the subsequent implication of nucleotides in the synthesis *de novo* of purine derivatives (7), prompted a series of investigations to determine whether nucleic acid derivatives more complex than purines and pyrimidines might also be involved in nucleic acid biosynthesis. It was expected that such knowledge would lead to an understanding of the steps in the biosynthetic pathways leading to nucleic acids.

The first evidence that larger molecules are indeed incorporated into nucleic acids of the rat was obtained in experiments in which the feeding of yeast nucleic acid uniformly labeled with N^{15} and the injection of a mixture of nucleotides from the same nucleic acid resulted in a pattern of labeling the purines of the tissue polynucleotides unlike that found after the administration of isotopic bases (8, 9). Subsequently, in a study of the metabolism, by the rat, of C^{14}-labeled nucleosides Lowy *et al.* found that, although adenosine is used in the synthesis of nucleic acids in the same

* The first report in this series dealt with the phosphates of nucleotides (1). This investigation was supported by funds from the National Cancer Institute, National Institutes of Health, Public Health Service (grant No. C-471), and from the Atomic Energy Commission (contract No. AT(30-1)-910). A preliminary report of a portion of this work has appeared (2).

† Present address, Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisconsin.

[1] The following abbreviations are used: PNA, pentose nucleic acid; DNA, deoxyribonucleic acid; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5′-phosphate.

XUM

manner as adenine, it is a much less effective precursor than is the free purine (10). They also found that guanosine is incorporated in the same pattern and to about the same small extent as is guanine.

After the experiment with nucleic acid-N^{15}, it was decided to isolate individual nucleotides from the uniformly labeled nucleic acid-N^{15} and to study their metabolism in the rat. The results of such studies with the purine nucleotides are presented in this paper. Before this experiment could be started, certain preliminary investigations were necessary. First, a method was required for the large scale isolation of nucleotides in good yield from the nucleic acid-N^{15}, and, in order that the isotopic compound should not be wasted, it was necessary that the method should allow for the preparation of all four nucleotides from a single sample of nucleic acid. Although procedures for the isolation of nucleotides by ion exchange methods had been reported, facilities were not available for large scale preparations; accordingly, a procedure was developed for the isolation of both purine and pyrimidine nucleotides from an alkaline hydrolysate of yeast nucleic acid. The details of this method are presented in this paper. It was also necessary to develop a method for the separation of the PNA and the DNA from the tissues and for the isolation from these of individual bases in sufficient quantity and in a form satisfactory for determination of N^{15}. The details of that method are also presented here.[2]

In addition to the determination of the extent of incorporation of the purine nucleotides into the nucleic acids of the rat, measurements were also made of the extent of labeling of the urinary allantoin and urea resulting from the metabolism of these nucleotides. Measurements were also made of the extent of incorporation of adenylic acid into muscle ATP and ADP, and, in addition, the ATP was degraded in such a way that the N^{15} content of the ring nitrogens and the amino nitrogen could be determined separately.

By such experiments it was possible to show that adenylic acid serves as a precursor of both the adenine and the guanine of the PNA of the rat and is approximately as effective as is adenosine. Unlike guanine and guanosine, which are used scarcely at all for nucleic acid synthesis by the rat, guanylic acid was found to be an effective precursor of PNA guanine. Both the amino nitrogen and purine ring nitrogens of muscle ATP were derived from the adenylic acid. There was extensive conversion of the purines of both of the administered nucleotides to urinary allantoin and definite conversion of each to urea.

EXPERIMENTAL

Isolation of Mononucleotides—10 gm. of uniformly labeled yeast nucleic acid (8) (3.34 atom per cent excess N^{15}) were dissolved in 50 ml. of 1 N

[2] This method has been referred to in a number of publications (11–14).

NaOH and were heated on the steam bath for 2 hours to produce a mixture of mononucleotides. The solution was brought to pH 8 by the addition of 6 N H_2SO_4, and a small amount of gummy residue was removed by filtration through Celite. The solution was brought to boiling and copper salts of purine nucleotides were precipitated according to the method of Laufer and Charney (15), by the addition of a solution made by mixing 50 ml. of copper sulfate solution (25 gm. of $CuSO_4 \cdot 5H_2O$ dissolved in 100 ml. of H_2O) and 12 ml. of sodium sulfite solution (28 gm. of $NaHSO_3$ dissolved in 100 ml. of H_2O). The mixture was stirred and boiled for 1 to 2 minutes and was then allowed to cool to room temperature. The precipitate of copper purine nucleotides was removed by centrifugation and washed once with warm water. The solution and washings were combined and set aside for the preparation of pyrimidine nucleotides.

Isolation of Purine Nucleotides—The precipitate was washed twice more, suspended in about 150 ml. of H_2O, and decomposed with H_2S. The CuS was removed by filtration, and H_2S was removed from the filtrate by aeration. The solution was concentrated *in vacuo* to about 20 ml., brought to pH 7 with concentrated NH_4OH, and ammonium guanylate precipitated by the addition of 40 ml. of ethanol. The ammonium guanylate was reprecipitated three times and was converted through the lead salt to the free acid according to the procedure of Buell and Perkins (16), except that the precipitation of silver guanylate was omitted. The solution containing guanylic acid was concentrated to about 5 ml. *in vacuo*, and ethanol was added to precipitate the free nucleotide. The guanylic acid was dried with ethanol and ether. Yield 0.310 gm.

$C_{10}H_{14}N_5O_8P \cdot 2H_2O$. Calculated, N 17.6, P 7.8; found, N 17.0, P 8.4

The solution containing the adenylic acid was concentrated *in vacuo* to 10 ml. to remove ethanol and was then diluted to 25 ml., after which it was made just acid to litmus with glacial acetic acid. Lead adenylate was prepared and decomposed in the same way the lead guanylate was. The solution containing adenylic acid was then concentrated *in vacuo* to 10 ml. and placed in the refrigerator for several days, whereupon a precipitate of crystalline adenylic acid was formed. It was recovered by filtration, washed with a very small amount of cold H_2O, ethanol, and ether, and dried in air. It was recrystallized once from water to yield 0.482 gm.

$C_{10}H_{14}N_5O_7P \cdot H_2O$.	Calculated.	N 19.2, P 8.5, C 32.9, H 4.4
	Found.	" 19.1, " 8.7, " 32.9, " 4.3

Isolation of Pyrimidine Nucleotides—The solution containing the pyrimidine nucleotides was heated to boiling, and a slight amount of white precipitate which formed was removed by filtration. Copper was removed from solution by treatment with H_2S, followed by filtration and aeration.

The solution was then concentrated *in vacuo* to 50 ml., and 1.4 ml. of concentrated H_2SO_4 were added. The solution was heated to boiling, and 15 gm. of phosphotungstic acid dissolved in hot 1 N H_2SO_4 were added to precipitate cytidylic acid according to the procedure of Loring *et al.* (17). Boiling was continued for 2 minutes, and the solution was then allowed to stand until it reached room temperature. It was then placed in the refrigerator, overnight. A precipitate of crystalline cytidylic acid phosphotungstate was recovered by centrifugation in the cold, washed with a small amount of cold dilute phosphotungstic acid, and converted to crystalline cytidylic acid as described previously (17). After recrystallization 0.588 gm. was obtained.

$C_9H_{14}N_3O_8P$. Calculated, N 13.0, P 9.6; found, N 12.8, P 9.8

The supernatant solution obtained after the removal of the cytidylic acid phosphotungstate was treated with 5 ml. of NH_4OH to precipitate ammonium phosphotungstate, which was removed by centrifugation. The supernatant solution was brought to pH 3 to 4 with 12 N NaOH, concentrated *in vacuo* to 20 ml., treated with 80 ml. of ethanol to precipitate inorganic salts, and filtered. The salts were dissolved in 20 ml. of hot water, the solution was chilled, 80 ml. of ethanol were added, and the salts were again removed by filtration. The two filtrates were combined, concentrated *in vacuo* to 20 ml., heated to boiling, and treated with an alcoholic solution containing 5 gm. of brucine. The brucine uridylate was recovered after the solution had remained 3 days in the refrigerator and was converted into diammonium uridylate as described by Levene and Bass (18). After recrystallization 0.550 gm. was obtained.

$C_9H_{19}O_9N_4P \cdot H_2O$. Calculated, N 14.9, P 8.3; found, N 14.7, P 8.6

The purity of the purine nucleotides was established by a comparison of their spectra with those of known compounds. Filter paper chromatography (isoamyl alcohol, 5 per cent citric acid, pH 9.6; an ascending chromatogram (19)) served as an additional characterization. It was found that adenylic acid was free of other purine or pyrimidine compounds, but that the guanylic acid was contaminated with a small amount (about 5 per cent) of guanine or guanosine. Since it has been shown that these compounds are incorporated only very slightly into the nucleic acids of the rat, it was felt that this small contamination would have no significant effect on the results.

Injection of Nucleotides—The adenylic and guanylic acids were administered by subcutaneous injection at a level of 0.4 mmole per kilo per day for 3 days[3] to adult male Sherman strain rats, which were maintained in

[3] In many of the tracer experiments reported from this laboratory the level of administration has been 0.2 mmole of isotopic compound per kilo of body weight per day

metabolism cages. Two rats, weighing 296 and 235 gm., were given a total of 254 mg. of the guanylic acid-N^{15}, and three rats, weighing 237, 237, and 285 gm., received a total of 332 mg. of the adenylic acid-N^{15}. At the end of the 3rd day, those animals which had received guanylic acid were sacrificed by ether anesthesia, whereas those which had received adenylic acid were killed by injections of urethane and magnesium sulfate, as previously described (3). The liver, kidneys, spleen, small intestines, and testes were removed and dehydrated (3). Leg and back muscles were quickly removed from those animals which had received adenylic acid and were frozen on dry ice. Urine was collected throughout the experiments, and allantoin and urea were isolated (3).

Isolation of Purines and Pyrimidines from Nucleic Acids of Tissues. (*a*) *Nucleic Acid Fractions*—The following modification of the Schmidt-Thannhauser procedure (22) was used for the isolation and separation of PNA and DNA. The dehydrated tissue was suspended in 10 per cent NaCl (20 ml. per gm. of tissue) and heated on the steam bath with constant stirring for 6 hours. The volume was maintained by occasional additions of water. The hot suspension was filtered by suction, with Whatman No. 1 filter paper and as large a Büchner funnel as practical, and the residue was washed with a small amount of hot 10 per cent NaCl. The filtrate and wash solution were combined and mixed with 2.5 volumes of ethanol. After standing several hours in the cold, the suspension was centrifuged, and the sodium nucleates were washed with ethanol, ether, and dried in air.

300 mg. of sodium nucleates were dissolved in 2 ml. of 1 N NaOH and allowed to stand at room temperature for 20 to 24 hours. Any insoluble material was removed by centrifugation. The solution was chilled in an ice bath and 1.0 ml. of ice-cold 6 N HCl and 2 ml. of ice-cold 5 per cent trichloroacetic acid were added to precipitate the DNA. The suspension was immediately centrifuged and yielded a somewhat opalescent solution, which contained the ribonucleotides, and a gummy precipitate which contained DNA. The residue was washed once with cold 1 per cent trichloroacetic acid, with ethanol, and with ether to give a dry powder of crude DNA. The precipitation and recovery of DNA are best carried out in the cold room.

The ribonucleotide solution was adjusted to pH 8 to 9 with a saturated solution of $Ba(OH)_2$, 2 volumes of ethanol were added, and after the suspension of barium ribonucleotides had remained in the cold for several hours the precipitate was recovered by centrifugation, washed with 70 per cent, and then with absolute alcohol and ether, and dried in air.

for 3 days. However, because the nucleotides used in these experiments are mixtures of the *a* (2′) and *b* (3′) isomers (20, 21), it was considered advisable to administer them at a level of 0.4 mmole per kilo per day in order that approximately 0.2 mmole of each isomer would be furnished.

(*b*) *Separation of Purines and Pyrimidines*—The purines and pyrimidines were liberated from the nucleic acid fractions by $HClO_4$ hydrolysis (23). For every 100 mg. of either crude DNA or barium ribonucleotides 2 ml. of 72 per cent perchloric acid were added and the suspension was heated on the steam bath for 45 minutes.[4] The black suspension was diluted with 2 volumes of H_2O and filtered through a sintered glass funnel covered with a thin pad of Celite. The residue was washed with water, and the filtrate and washings were combined. The solution was brought to pH 3 to 4 by the addition of 10 N KOH, and the precipitated $KClO_4$ was removed by filtration and washed with a very small amount of cold water. To the filtrates were added 10 ml. of 10 per cent $AgNO_3$ and then 10 N NaOH, dropwise, to precipitate purines and pyrimidines as silver salts. Completeness of precipitation is indicated by the production of a permanent precipitate of dark brown silver oxide, at which point the suspension is pH 7 or greater. The precipitate of silver salts of purines, pyrimidines, and phosphate was recovered by centrifugation and washed with water. It was decomposed by the addition of 10 ml. of 1 N HCl and by heating the mixture on the steam bath for 3 to 4 minutes with occasional stirring. The AgCl was removed by centrifugation, treated with an additional 10 ml. of hot 1 N HCl, and again centrifuged. The two HCl solutions were concentrated *in vacuo* to dryness. The residue was dissolved in H_2O and again taken to dryness. The residue was now taken up in 25 to 50 ml. of H_2O, care being taken that any insoluble material, chiefly guanine, was scraped from the walls of the flask and brought into suspension. The solution was brought to pH 12 by the addition of the calculated amount of 1 N NaOH, and any precipitate which formed was removed by centrifugation and discarded. The number of optical density units[5] in the solution is determined by measuring the optical density of a suitably diluted acidified aliquot.

For the separation of the bases a modification of an ion exchange procedure described by Cohn (24) was used.[6] In Cohn's method ammonia buffers were employed for the elution of the bases, but their use in the separation of compounds which were to be analyzed for N^{15} had to be avoided. Instead, a system utilizing solutions of NaOH and NaCl was devised.

[4] A temperature of nearly 100° must be maintained. A cytosine derivative "X" (probably a cytidylic acid), eluted prior to adenine (Fig. 1) has been encountered, and it is now known that this occurs only when the temperature is not sufficiently high during the hydrolysis, or when the perchloric acid is excessively diluted.

[5] Optical density units are defined as the optical density of the solution, at 260 mμ and pH 2, times the volume of the solution in ml.

[6] The authors wish to express their thanks to Dr. Waldo E. Cohn, with whom the problem was discussed.

For the separation of the bases a column of Dowex 1[7] (OH^- form) was prepared by passing 5 ml. of 1 N NaOH over a column of Dowex 1 (Cl^- form, 200 to 400 mesh) which had been prepared as described by Cohn (25). Water was forced through the column until the eluent was neutral. The alkaline solution of bases was added to the column, under pressure, and the resin was again washed until the eluent was no longer alkaline.

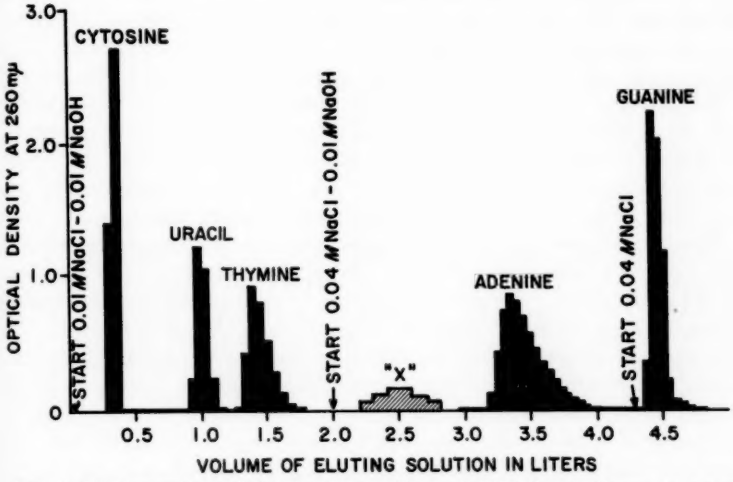


FIG. 1. Elution pattern of five bases from Dowex 1 (OH^- form), 10 cm. × 1.2 sq. cm. Rate of flow, 1.0 ml. per minute. The mixture contained 40 μmoles each of cytosine and guanine, 25 μmoles each of adenine and thymine, and 20 μmoles of uracil. Recoveries varied from 98 to 102 per cent. If present, "X" (see foot-note 4) is eluted at the point indicated.

A column, 0.8 sq. cm. × 10 cm. in size, will handle 800 to 1200 optical density units (about 100 μmoles) of mixed bases. If more material is to be separated, a column of proportionately larger cross-sectional area should be used. The pyrimidines and purines were eluted according to pattern shown in Fig. 1. After adenine has been removed, guanine may be eluted with the same eluent or, more rapidly, with 0.04 N NaCl alone.

Those fractions containing the bulk of each base were combined, diluted with an equal volume of water, made 0.01 N with NaOH, and passed over a concentration column of Dowex 1 (OH^- form) 2 sq. cm. × 1 to 2 cm. The column was washed with H_2O until the effluent was no longer alkaline, and the purine or pyrimidine was eluted with 20 to 30 ml. of 1 N HCl. The acid solution was concentrated to dryness *in vacuo*, and the residue re-

[7] National Aluminate Corporation, Chicago, Illinois.

peatedly taken up in H_2O and concentrated. The bases were finally dissolved (or suspended in the case of guanine) in H_2O and were ready for the determination of N^{15}.

Evaluation of Isolation Procedure—A study was made of the effectiveness of the extraction from tissues and the recovery of the nucleic acids. Two weighed samples of dehydrated rat viscera were extracted with hot 10 per cent sodium chloride, one sample for 4 hours, the other for two 5 hour periods, and the nucleic acids were precipitated as described. The tissue residues and the sodium nucleates were dried in air and weighed. Each supernatant solution, from which the sodium nucleate was precipitated, was concentrated on the steam bath to remove alcohol, diluted to volume, and the optical density at 260 mμ was measured against a blank prepared by mixing 1 volume of 10 per cent NaCl with 2.5 volumes of ethanol and concentrating and diluting in the same way. Thus, the number of optical density units not precipitated by alcohol could be calculated.

Weighed samples of the same dehydrated tissues were extracted first with cold and then with hot 5 per cent trichloroacetic acid as described by Schneider (26). Weighed samples of the tissue residues and sodium nucleates were extracted with hot 5 per cent trichloroacetic acid. The extracts were made 0.4 N with respect to H_2SO_4 and heated 1 hour in a boiling water bath to decompose the trichloroacetic acid. They were then diluted to volume. The optical density at 260 mμ of each solution was read against a blank of 5 per cent trichloroacetic acid which had been acidified, heated, and diluted exactly as the test sample. In this way it was possible to calculate the number of optical density units in each fraction (Table I).

Isolation of ATP and Inosinic Acid—The frozen muscle (98 gm.) was treated as described by LePage (27) up to the first precipitation of the barium salt. This crude barium ATP was recovered by centrifugation and dried with alcohol and ether. The yield was 502 mg., of which 170 mg. were dissolved in cold 0.1 N HCl and treated with 0.5 M Na_2SO_4 to precipitate $BaSO_4$ completely, which was removed by centrifugation. The solution was immediately adjusted to pH 6 with 1 N NaOH and fractionated by the procedure of Cohn and Carter (28). The mixture was found to contain 85 per cent ATP, 15 per cent ADP, and little AMP. The main fractions of each nucleotide were combined, brought to pH 6.8 with 1 N NaOH, and treated with 1 M barium acetate and 2 volumes of alcohol to precipitate the barium salts of ATP or ADP, which were recovered by centrifugation, washed with alcohol and ether, dried in air, and analyzed for N^{15} content.

200 mg. of the first crude barium ATP were dissolved in 7 ml. of 0.1 N HCl and converted to AMP by $Ba(OH)_2$ hydrolysis as described by Kerr

(29). The AMP was precipitated with mercuric acetate, the mercury salt of AMP was decomposed, and the aerated solution was fractionated over Dowex 1 (28). 91 per cent of the material was recovered as AMP. The fractions containing AMP were combined, adjusted to pH 7 to 8 with 1 N NaOH, and passed over a column of Dowex 1 (Cl^- form), 0.8 sq. cm. × 1 cm. The AMP was eluted with 0.1 M NaCl-0.01 M HCl, and three fractions (6.5 ml.) containing most of the AMP were combined. To convert the AMP to inosinic acid (30) there were added to this solution 0.8 ml. of 0.7 M potassium acetate buffer, pH 6, and 3.0 ml. of a solution of rabbit muscle adenylic acid deaminase (31, 32) which had been dialyzed against the potassium acetate buffer to remove NH_3. This system was incubated at 37°, and the progress of hydrolysis was followed as described by Kalckar and Rittenberg (30). After 3 hours, when hydrolysis was complete, 1.5 ml. of 20 per cent trichloroacetic acid were added, the precipitate was removed by centrifugation, the solution was adjusted to pH 4 to 5, and 1 ml. of 20 per cent mercuric acetate was added to precipitate inosinic acid, which was removed by centrifugation and analyzed for N^{15}. The solution was made strongly alkaline and the ammonia from the deamination of the AMP was distilled into HCl and also analyzed for N^{15}. A blank, containing enzyme and buffer, was treated in the same way as the test solution and was found to contain practically no ammonia.

Results

In Table I are presented results showing the effectiveness of the extraction and recovery of nucleic acids from rat tissues. Not more than 4 per cent of the material which absorbs at 260 mμ remained unextracted by hot NaCl solution. It should be noted that this is not a method specific for the determination of nucleic acids, but that other materials which absorb at 260 mμ undoubtedly will be extracted with trichloroacetic acid, and this value for unextracted nucleic acid must be considered as a maximal value. It can also be seen that the amount of material extracted by cold trichloroacetic acid is a large fraction of that unprecipitated by alcohol, and it seems likely that the bulk of this unprecipitated material may be accounted for by the "acid-soluble fraction" of the tissues. The overall recovery of sodium nucleates from tissue exceeds 92 per cent. The demonstration that more than one type of PNA (33, 34) and of DNA (13) exists in tissues makes it imperative that the isolation procedures shall not involve fractionation of the nucleic acids when a study of the metabolism of the total nucleic acids of a tissue is intended. Since the method of extraction described here results in almost complete recovery of the nucleic acids, extensive fractionation is known to be avoided.

The preparations of DNA and barium nucleotides from the PNA were

assayed by the ion exchange separation of five bases (Fig. 1). Contamination of the DNA by ribose nucleic acid was negligible, as shown by the absence of uracil in the DNA hydrolysates. That of PNA by DNA was 0 to 3 per cent, as indicated by the occasional presence of some thymine in the PNA hydrolysates.

In Table II are presented results of studies on the incorporation of the purine nucleotides. It can be seen that adenylic acid, like adenine and adenosine, is a precursor of both the adenine and the guanine of the PNA. The small concentration of N^{15} found in the PNA pyrimidines and in all of the DNA purines and pyrimidines probably arose from isotopic "body pool ammonia" and would tend to mask any small direct conversion of

TABLE I

Extraction of Nucleic Acids from Tissues

Time of extraction, *hrs.*	4	5 + 5
Weight of tissue extracted, *gm.*	1.00	1.00
" " residue, *gm.*	0.625	0.766
" " sodium nucleates, *gm.*	0.133	0.129
	Optical density units	
From total tissue (cold trichloroacetic acid)*	280	320
In alcoholic supernatant	505	363
From total tissue (hot trichloroacetic acid)*	1668	1618
" " residue†	56	56
" " sodium nucleates*	1548	1491

* Calculated from determination on a 25 mg. aliquot.
† Calculated from determination on a 300 mg. aliquot.

adenylic acid into these compounds. That extensive labeling of body pool ammonia did occur is shown by the reasonably high level of isotope in urinary urea. From the fact that the allantoin is extensively labeled, it may be deduced that considerable deamination of the adenylic acid, or one of its derivatives, had occurred.

The sodium nucleate obtained in the guanylic acid experiment was divided into two parts, each of which was processed separately. The identity of the two sets of results attests to the validity of these procedures for the preparation of samples of purines and pyrimidines, particularly for studies with N^{15}. Guanylic acid, unlike guanine and guanosine, is well incorporated into the guanine of the PNA. There is no evidence for conversion of guanylic acid to PNA adenine, although here again the general labeling, presumably from the amino groups via "body pool ammonia," would obscure a small amount of conversion. The DNA purines and pyrimidines all contain some excess N^{15}, but in both isolations the isotope content of the guanine is significantly higher than that of the other bases,

indicating that guanylic acid is utilized directly for the synthesis of DNA guanine. Allantoin was derived from guanylic acid to about the same extent that it was from adenylic acid. However, guanylic acid was converted to urea less effectively than was adenylic acid.

Adenylic acid is well incorporated into the ADP and ATP of muscle. It is noteworthy that the inosinic acid and the ammonia which are ob-

TABLE II

Incorporation of Purine Nucleotides

	Adenylic acid		Guanylic acid		
	Atom per cent N^{15} excess*	Relative isotope content†	Atom per cent N^{15} excess*		Relative isotope content†
Injected nucleotide	3.18	100	3.13		100
PNA adenine	0.120	3.8	0.010	0.009	
" guanine	0.040	1.3	0.067	0.068	2.2
" cytosine	0.010		Lost	0.005	
" uracil	0.009		0.012	Lost	
DNA adenine	0.016		0.005	0.004	
" guanine	0.008		0.013	0.012	0.41
" cytosine	0.007		0.007	0.007	
" thymine	0.014		0.007	0.005	
Muscle ATP	0.044	1.4			
" ADP	0.043	1.4			
" inosinic acid‡	0.041	1.3			
" NH_3‡	0.039	1.2			
Urinary allantoin	0.596		0.570		18.2
" urea	0.027		0.015		0.48

* Consolidated-Nier, model 21–201, ratio mass spectrometer; probable error ±0.001.

† Relative isotope content $= \frac{\text{atom \% excess in isolated compound}}{\text{atom \% excess in administered compound}} \times 100.$

‡ Isolated from the ATP.

tained from ATP were labeled to approximately the same extent, which indicates that during the incorporation of adenylic acid into ATP the purine ring nitrogen as well as the amino nitrogen is used.

DISCUSSION

It has been clearly demonstrated that adenine and adenosine are utilized by the rat as precursors of both polynucleotide adenine and guanine. Thus, when adenosine was given to rats at a level of 0.2 mmole per kilo per day for 3 days, it was found that 2.4 per cent of the adenine and 0.61 per cent of the guanine of the PNA were derived from the nucleoside (10).

The present results show that yeast adenylic acid (containing the *a* and *b* isomers) furnished at twice this level is approximately twice as extensively incorporated into both adenine and guanine of PNA. This suggests that the nucleoside and nucleotide can serve equally well as precursors and furnishes no information as to which of the two might be the more immediate precursor. However, neither adenylic acid nor the adenosine is nearly as well utilized as is adenine, since in several experiments when the purine was given at the 0.2 mmole level the poorest incorporation observed was more than twice as great as that of the adenosine (3, 35). Thus, the degradation of adenylic acid, or adenosine, to adenine prior to incorporation cannot be ruled out, and the exact rôle of the nucleotide in the biosynthesis of nucleic acids remains obscure. It is true, however, that calculations of the relative extent of incorporation of several compounds may be misleading when information concerning the concentration of the precursors at the site of metabolism is lacking. Some of the factors which can influence such concentrations and which are difficult or impossible to evaluate are the normal concentration of the precursors in the tissues, the extent of enzymatic degradation of the compounds, and the extent to which the precursors participate in other biosynthetic processes. For example, in the rat, the wide-spread distribution of phosphatases, adenylic acid deaminase and adenosine deaminase, coupled with the apparent low level of enzymes which attack adenine might well allow the purine to arrive at and remain at the site of nucleic acid biosynthesis long after the nucleotide and nucleoside had been destroyed.

Only when adenine was given at a very high level (1.5 mmoles per kilo per day), was it possible to demonstrate its incorporation into muscle ATP (3),[8] and in the adenosine experiment (10) it was found that only 0.17 per cent of the muscle ATP had been derived from the nucleoside. However, the present results show that 1.4 per cent of both muscle ADP and ATP was formed from adenylic acid. Furthermore, it was found that the purine ring nitrogens and the 6-amino nitrogen had been derived to about the same extent from the injected nucleotide, which strongly suggests that no extensive deamination occurred prior to or following incorporation into the ATP. This is to be contrasted with the fact that Kalckar and Rittenberg (30) observed that the 6-amino nitrogen of muscle ATP was renewed much more rapidly from ammonium citrate than were the purine ring nitrogens.

Guanine (4) and guanosine (10) were only very slightly incorporated into the PNA guanine in the rat. The finding that guanylic acid is well incorporated is therefore highly significant, and the interpretation was possible (39) that guanylic acid is incorporated as a unit into nucleic acids of the

[8] It has recently been found that adenine is well incorporated into adenine nucleotides in the acid-soluble fraction of mouse viscera and pigeon liver preparations (36–38).

rat. Subsequent experiments with labeled phosphate (1) have shown that this cannot be true, but that, throughout the pathway of the incorporation of the guanine of guanylic acid, units at least as complex as guanylic acid must be involved.

In the experiment with guanylic acid, in which the isolations were carried out in duplicate, a definite incorporation into the guanine of DNA was noted. If allowance is made for the general labeling of all of the purines and pyrimidines of both PNA and DNA (undoubtedly due to utilization of $N^{15}H_3$ liberated by deamination of some of the guanylic acid), it is possible to calculate that the ratio of incorporation of guanylic acid into the PNA and DNA is approximately 10:1, although a similar conclusion cannot be drawn from the adenylic acid experiment. This is considerably lower than the ratios found for the incorporation of adenine into PNA and DNA (40).

It is of interest to note that, in the experiment in which nucleic acid-N^{15} was hydrolyzed to yield a mixture of nucleotides and was injected into rats in amount to furnish approximately 0.4 mmole each of adenylic acid and guanylic acid per kilo per day, it was found that 3.7 per cent of the PNA adenine and of the guanine had each been formed from the nucleotide mixture. In confirmation, if the incorporation of the two nucleotides measured separately in the present experiments is added, it is seen that 3.8 per cent of the adenine and 3.5 per cent of the guanine were derived from the two nucleotides.

SUMMARY

1. A method is described for the preparation of four ribonucleotides, in good yield, from a single sample of yeast nucleic acid.

2. A procedure is described for the isolation of individual purines and pyrimidines from the PNA and from the DNA of tissues.

3. It was found that yeast adenylic acid (*a* and *b* isomers) uniformly labeled with N^{15} is incorporated into adenine and guanine of the PNA of rat viscera to about the same extent that adenosine was, but not nearly as well as was adenine.

4. The adenylic acid was also found to be an effective precursor of the purine ring nitrogens and the 6-amino nitrogen of muscle ATP in the rat.

5. Yeast guanylic acid (*a* and *b* isomers), unlike guanine and guanosine, is well utilized for the synthesis of PNA guanine in the rat.

The authors wish to thank Dr. George Bosworth Brown for continued interest and discussions, the late Mr. Roscoe C. Funk, Jr., for the microanalyses, and Mr. Arthur Brandt for technical assistance in some of the preparations.

BIBLIOGRAPHY

1. Roll, P. M., Weinfeld, H., and Brown, G. B., *Biochim. et biophys. acta*, **13**, 141 (1954).
2. Roll, P. M., and Weliky, I., *Federation Proc.*, **10**, 238 (1951).
3. Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., *J. Biol. Chem.*, **172**, 469 (1948).
4. Balis, M. E., Marrian, D. H., and Brown, G. B., *J. Am. Chem. Soc.*, **73**, 3319 (1951).
5. Bendich, A., Getler, H., and Brown, G. B., *J. Biol. Chem.*, **177**, 565 (1949).
6. Plentl, A. A., and Schoenheimer, R., *J. Biol. Chem.*, **153**, 203 (1944).
7. Greenberg, G. R., *J. Biol. Chem.*, **190**, 611 (1951).
8. Di Carlo, F. J., Schultz, A. S., Roll, P. M., and Brown, G. B., *J. Biol. Chem.*, **180**, 329 (1949).
9. Roll, P. M., Brown, G. B., Di Carlo, F. J., and Schultz, A. S., *J. Biol. Chem.*, **180**, 333 (1949).
10. Lowy, B. A., Davoll, J., and Brown, G. B., *J. Biol. Chem.*, **197**, 591 (1952).
11. Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **195**, 257 (1952).
12. Conzelman, G. M., Jr., Mandel, H. G., and Smith, P. K., *J. Biol. Chem.*, **201**, 329 (1953).
13. Bendich, A., Russell, P. J., Jr., and Brown, G. B., *J. Biol. Chem.*, **203**, 305 (1953).
14. Dancis, J., and Balis, M. E., *J. Biol. Chem.*, **207**, 367 (1954).
15. Laufer, L., and Charney, J., U. S. patent 2,379,914 (1945).
16. Buell, M. V., and Perkins, M. E., *J. Biol. Chem.*, **72**, 21 (1927).
17. Loring, H. S., Roll, P. M., and Pierce, J. G., *J. Biol. Chem.*, **174**, 729 (1948).
18. Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York, 220 (1931).
19. Carter, C. E., *J. Am. Chem. Soc.*, **72**, 1466 (1950).
20. Khym, J. X., Doherty, D. G., Volkin, E., and Cohn, W. E., *J. Am. Chem. Soc.*, **75**, 1262 (1953).
21. Cavalieri, L. F., *J. Am. Chem. Soc.*, **75**, 5268 (1953).
22. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).
23. Marshak, A., and Vogel, H. J., *J. Biol. Chem.*, **189**, 597 (1951).
24. Cohn, W. E., *Science*, **109**, 377 (1949).
25. Cohn, W. E., *J. Am. Chem. Soc.*, **72**, 1471 (1950).
26. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
27. LePage, G. A., *Biochem. Preparations*, **1**, 5 (1949).
28. Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72**, 4273 (1950).
29. Kerr, S. E., *J. Biol. Chem.*, **139**, 131 (1941).
30. Kalckar, H. M., and Rittenberg, D., *J. Biol. Chem.*, **170**, 455 (1947).
31. Schmidt, G., *Z. physiol. Chem.*, **179**, 243 (1928).
32. Kalckar, H. M., *J. Biol. Chem.*, **167**, 445 (1947).
33. Marshak, A., *J. Biol. Chem.*, **189**, 607 (1951).
34. Griffin, A. C., Davis, W. E., Jr., and Tifft, M. O., *Cancer Res.*, **12**, 707 (1952).
35. Marrian, D. H., Spicer, V. L., Balis, M. E., and Brown, G. B., *J. Biol. Chem.*, **189**, 533 (1951).
36. Bennett, E. L., *Biochim. et biophys. acta*, **11**, 487 (1953).
37. Scarano, E., *Nature*, **172**, 949 (1953).
38. Goldwasser, E., *Biochim. et biophys. acta*, **13**, 341 (1954).
39. Brown, G. B., Roll, P. M., and Weinfeld, H., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **2** (1952).
40. Furst, S. S., Roll, P. M., and Brown, G. B., *J. Biol. Chem.*, **183**, 251 (1950).

THE UTILIZATION OF NUCLEOTIDES BY THE MAMMAL

III. ISOMERIC PURINE NUCLEOTIDES LABELED WITH C^{14}*

By HERBERT WEINFELD,† PAUL M. ROLL,‡ AND GEORGE BOSWORTH BROWN

(From the Laboratories of the Sloan-Kettering Division of Cornell University Medical College, New York, New York)

(Received for publication, September 28, 1954)

In the studies of N^{15}-labeled purine nucleotides as precursors of pentose nucleic acids (PNA) in the rat (3), it was found that the purine of yeast adenylic acid was incorporated into nucleic acid adenine of the viscera and converted to nucleic acid guanine; the purine of yeast guanylic acid served as a precursor of nucleic acid guanine only. Each purine nucleotide administered was presumably a mixture of the *a* and *b* isomers (4, 5), since they were obtained by alkaline hydrolysis of yeast ribonucleic acid.

In this study the individual isomers, labeled with C^{14} in the purine moieties, have been investigated. The compounds studied were adenylic acid *a* (AMP-2′) (6, 7), adenylic acid *b* (AMP-3′) (6, 7), adenosine-5′-phosphate (AMP-5′), and the *b* (presumably 3′) isomer of guanylic acid. Observations were also made on the utilization of these nucleotides for formation of muscle adenosine polyphosphates.

EXPERIMENTAL

Preparation of Nucleotides—The C^{14}-labeled adenylic acids *a* and *b* and guanylic acid *b* were prepared biosynthetically. The yeast *Torulopsis utilis*, which incorporates adenine into PNA and converts adenine to PNA guanine (8), was grown in a medium adapted from Schultz and Atkin (9). It consisted of the recommended salt solution, 100 ml., and potassium citrate buffer, pH 5.2, 16 ml., to which were added 20 gm. of glucose and 170 ml. of distilled water. The solution was autoclaved in a 1 liter Erlenmeyer flask for 20 minutes at 10 pounds pressure and supplemented with 15 ml. of a vitamin solution (9), including riboflavin, 50 γ per ml., prepared with sterile distilled water. Adenine-8-C^{14}, of specific activity of

* This investigation was supported by funds from the National Cancer Institute, National Institutes of Health, Public Health Service (grant No. C-471), from the Atomic Energy Commission (contract No. AT(30-1)-910), and from the Nutrition Foundation, Inc. Preliminary reports of portions of this work have appeared (1, 2).

† Present address, Institute of Industrial Medicine, New York University-Bellevue Medical Center, New York.

‡ Present address, Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisconsin.

1.6×10^6 c.p.m. per μmole, was added in 35 ml. of sterile distilled water. Yeast grown for 24 hours at 30° on two slants of Sabouraud's glucose agar was suspended in 10 ml. of sterile 0.9 per cent NaCl and added to the medium. The flask was plugged with cotton and the contents swirled on a rotary shaker for 24 hours at 25°. Two runs involving a total of 9.6 mg. (71 μmoles) of adenine-8-C^{14} were made. The yeast was harvested by centrifugation, washed three times on the centrifuge with ice-cold 5 per cent trichloroacetic acid, absolute alcohol, three times with hot 3:1 alcohol-ether, and finally with ether. The air-dried material was suspended in 20 volumes of 1 N NaOH for 20 hours at room temperature, followed by a 4 hour period at 37°. The mixture was centrifuged and the residue was discarded. The clear supernatant solution of the ribomononucleotides was adjusted to pH 8 by addition of the cation exchange resin Zeo-Rex[1] (8) and separated from the resin by decantation. After combining several water washings of the resin with the nucleotide solution, HCl was added to pH 2 to 3, and the solution was clarified by centrifugation. Following pH adjustment to approximately 7 with NaOH, the nucleotides were fractionated on a Dowex 1[2] chloride ion exchange column, 200 to 400 mesh, by the method of Cohn (5) to obtain the adenylic acids *a* and *b* and guanylic acids *a* and *b*; 0.005 N HCl was used to elute the guanylic acids. To concentrate the compounds they were absorbed on beds of Dowex 1 chloride, 1 cm. × 0.8 sq. cm., eluted with approximately 20 ml. of ice-cold 0.1 N HCl, and immediately neutralized. Aliquots were diluted in 0.01 N HCl and read at 250, 260, 280, and 290 mμ in the spectrophotometer. The optical density ratios 250:260, 280:260, and 290:260 were in satisfactory agreement with published values (10). Paper chromatography of the radioactive adenylic acid *a* or *b* in 5 per cent disodium phosphate-isoamyl alcohol (4) revealed that less than 1 per cent of adenine, adenosine, or the other isomer was present. Recycling an aliquot of the guanylic acid *b* preparation over Dowex 1 chloride in the fractionation procedure also indicated that guanine, guanosine, and guanylic acid *a* were absent. The yields were 25 to 35 μmoles of each nucleotide from a total of 5 gm. of dried yeast, with specific activities of 4 to 5×10^5 c.p.m. per μmole. The compounds were stored in the frozen state.

In order to ascertain the distribution of C^{14} between the purine and ribose moieties of the mononucleotides, a sample of the adenylic acid *b*-C^{14} preparation was mixed with commercial adenylic acid *b* (Schwarz Laboratories, Inc.) and the C^{14} activity of the mixture determined. It was then hydrolyzed with 1 N HCl on the steam bath for 1.5 hours. After removal of HCl *in vacuo* the adenine was isolated as the silver salt, freed of

[1] The Permutit Company, New York.
[2] National Aluminate Corporation, Chicago, Illinois.

silver, and the C^{14} activity of the purine determined. The specific activities for the intact nucleotide and the free base were 9180 and 8690 c.p.m. per μmole, respectively, and thus, within experimental error, the isotope resided solely in the purine moieties.

Isotopically labeled AMP-5′ was prepared from synthetic adenosine-8-C^{14} (11) by enzymatic phosphorylation with adenosinetriphosphate (ATP) by the method of Kornberg and Pricer (12). The AMP-5′ was separated from adenosinediphosphate (ADP) and ATP by fractional elution from Dowex 1 chloride (13), and a concentrated neutral solution was prepared as described above. The spectrophotometric constants (10) were satisfactory.

A sample of yeast guanylic acid was fractionated by ion exchange chromatography (5), and a quantity of guanylic acid *b*, although but little guanylic acid *a*, was obtained. It was concentrated as described above; alcohol was added to the final neutralized solution, which was then chilled in the refrigerator overnight, and the precipitate was collected, washed with alcohol and ether, and dried in air. The spectrophotometric constants (10) of the product indicated that all ultraviolet-absorbing material present was guanylic acid, and recycling of a sample over Dowex 1 chloride showed that guanylic acid *a* was absent.

Subcutaneous Injection of Radioactive Nucleotides—Solutions for administration of the compound to the experimental animals were prepared by mixing the labeled nucleotides with the corresponding non-isotopic compounds to achieve the desired dosage levels and specific activities. Adenylic acids *a* and *b*, chromatographically free of the other isomer, were obtained from the Schwarz Laboratories, Inc. AMP-5′ was a product of the Sigma Chemical Company. The spectrophotometric constants of these preparations agreed well with published values (10). Guanylic acid *b* was the preparation described above. Concentrations were determined with molar extinction coefficients at 260 mμ of 14,200 for the adenylic acids in 0.01 N HCl and 11,800 for guanylic acid *b* in distilled water (10).

Single adult male Sherman strain rats each received one of the nucleotides by subcutaneous injection at a level of 0.2 mmole per kilo of body weight per day for 3 days. This dosage regimen has been used in a series of previous experiments with heavy isotopes (3, 14–16) and has been adhered to (17) to permit certain comparisons between the series of derivatives. The animals were fed Purina chow and tap water *ad libitum*. About 24 hours after the last injection, they were sacrificed under urethane anesthesia with 25 per cent $MgSO_4$ solution in small intraperitoneal injections. The livers, washed small intestines, spleen, kidneys, testes, and lungs were frozen in dry ice-alcohol. Hind leg and back muscles were removed and frozen in dry ice.

Isolation of Nucleic Acids—The pooled internal organs were homogenized in alcohol, suspended in boiling alcohol, washed with ether, and dried in air. Sodium nucleates were extracted and separated into deoxypentose nucleic acid (DNA) and barium PNA mononucleotides (3). The barium nucleotides were dissolved in ice-cold 0.1 N HCl, and barium was removed by the addition of Na_2SO_4. The nucleotide solution was adjusted to pH 6, and the individual PNA nucleotides were separated by ion exchange chromatography (5). Pyrimidine nucleotides were evaporated to dryness. Purine nucleotides were either evaporated to dryness and hydrolyzed in 1 N HCl for 1.5 hours on the steam bath or, alternatively, absorbed on small beds of Dowex 1 chloride, eluted with 1 N HCl, and hydrolyzed in the acid solution. After removal of acid *in vacuo*, the purines were isolated by absorbing them from alkaline solution (pH 12) on Dowex 1 in the hydroxyl form, eluting with 1 N HCl, and concentrating to dryness *in vacuo*, with several additions of water to assure complete removal of acid. DNA was hydrolyzed with perchloric acid, and the bases were obtained by ion exchange chromatography (3). Uracil was absent, which indicated that the DNAs were uncontaminated with PNA.

Muscle Adenosine Polyphosphates—Barium ATP was isolated from frozen muscle according to LePage (18). The barium salt was dissolved in ice-cold 0.1 N HCl and barium removed by the addition of Na_2SO_4. The resulting solution was neutralized and passed over Dowex 1 chloride and the absorbed compounds eluted as described for the separation of AMP-5′, ADP, and ATP (13). A fraction corresponding to ATP was not obtained in the experiment with adenylic acid *b*. The ADP and ATP fractions were characterized by spectrophotometric constants (10). Adenine was isolated from these fractions as described for the PNA nucleotides.

Determination of Radioactivity—The compounds isolated from the animals were dissolved in water and the solutions filtered. Concentrations and purity were determined by ultraviolet absorption spectroscopy. Aliquots containing 0.0200 to 0.100 μmole of the injected compounds or 0.300 to 2.00 μmoles of the isolated compounds were transferred to 10 sq. cm. aluminum planchets and evaporated to dryness under a heat lamp. The radioactivities were determined in an internal Geiger-Müller flow counter (Radiation Counter Laboratories, Inc., mark 12, model 1, helium-isobutane gas) and are expressed as counts per minute per micromole. No coincidence corrections were necessary. Maximal standard counting errors were as follows: injected nucleotides, 2 per cent; PNA purines in the adenylic acid *a* and *b* experiments, 3 per cent; PNA adenine and guanine in the AMP-5′ experiment, 6 and 10 per cent, respectively; PNA adenine and PNA guanine in the guanylic acid *b* experiment, 9 and 2 per cent, respectively; adenine of the muscle fractions in the adenylic acid *a* and *b* ex-

periments, 5 per cent; DNA purines in the adenylic acid *a* and *b* experiments and DNA guanine in the guanylic acid *b* experiment, 7 per cent. The activities of all other purines of DNA, pyrimidines, and pyrimidine nucleotides and the adenine of ATP in the AMP-5′ and guanylic acid *b* experiments were very low, and the counting errors in these cases approach 100 per cent. The specific activities presented in Table I are the average

TABLE 1

Incorporation of the Purine Moieties of Nucleotides by the Rat

Subcutaneous injection at 0.2 mmole per kilo of body weight per day for 3 days.

Compounds isolated	Nucleotide administered							
	Adenylic acid *a*		Adenylic acid *b*		Adenosine-5′-phosphate		Guanylic acid *b*	
	C.p.m. per μmole, 2.26×10^4	RSA*	C.p.m. per μmole, 2.28×10^4	RSA	C.p.m. per μmole, 3.31×10^3	RSA	C.p.m. per μmole, 2.00×10^4	RSA
PNA cytidylic acid	1	0	2	0.01			0	0
" uridylic acid							1	0.01
" adenylic acid *a*†	423	1.87	443	1.94	36	1.09	17	0.09
" " " *b*†	431	1.91	420	1.84	31	0.94	18	0.09
" guanylic " *a*†	146	0.65	154	0.68	14	0.42	206	1.03
" " " *b*†	139	0.62	160	0.70	14	0.42	210	1.05
DNA cytosine							0	0
" thymine					0	0	0	0
" adenine	36	0.16	37	0.16	0	0	1	0.01
" guanine	12	0.05	16	0.07	0	0	30	0.15
ADP†	62	0.27	133	0.58				
ATP†	69	0.31			2	0.06	2	0.01

* $\frac{\text{Specific activity of isolated compound in c.p.m. per } \mu\text{mole}}{\text{Specific activity of administered compound in c.p.m. per } \mu\text{mole}} \times 100.$

† Radioactivity of the base determined.

of those from several samples of various sizes differing by 5 per cent or less from the average.

RESULTS AND DISCUSSION

It is apparent (Table I) that the purines of the isomeric injected adenylic acids, *a* and *b*, were about equally well utilized as sources of PNA and of DNA purines. Those isomers of adenylic acid were apparently 2 and 1½ times as effective as was AMP-5′ as sources of PNA adenine and guanine, respectively. They were considerably more effective with regard to DNA purines. In the absence of precise knowledge of "pool" sizes and the

relative susceptibilities to catabolism, a valid comparison cannot be made between adenylic acids *a* and *b* and AMP-5′ with respect to their effectiveness as nucleic acid precursors on the basis of the specific activities of the *injected* nucleotides.

The adenine of the muscle ATP and ADP fractions was renewed to the extent of 0.27 to 0.58 per cent in the experiments with adenylic acids *a* and *b*. The adenine of muscle ATP in the AMP-5′ experiment would have exhibited an activity of 9 c.p.m. per μmole had a renewal of as much as 0.27 per cent occurred. Although the recorded value of 2 c.p.m. per μmole is subject to a statistical error approching 100 per cent, there remains an indication that adenylic acids *a* and *b* are better utilized for formation of muscle adenosine polyphosphates than is AMP-5′. Reasons for the apparently superior utilization of the *a* and *b* isomers for the formation of these adenosine-5′-phosphate fractions may involve the presence of as yet uncharacterized adenine containing components of the fractions studied. In this connection it should be recalled that in experiments with adenosine-8-C^{14} (17) renewal of the total adenine of the muscle extract was greater than that of the ATP-adenine. It remains possible that the adenine of the *a* and *b* isomers does not pass through free AMP-5′ on the pathway of muscle ADP and ATP biosynthesis and that it also may not do so on the pathway of polynucleotide biosynthesis.

Earlier studies with adenosine-8-C^{14} (17) led to renewals of 2.4 and 0.61 per cent in the PNA adenine and guanine, respectively. Experiments with adenine yielded renewals of 4.0 (16), 5.4 (14), and 12.8 (16) per cent in polynucleotide adenine and 1.3 (16), 3.2 (14), and 5.2 (16) per cent in polynucleotide guanine. Those compounds were administered to rats at the same dose level and over the same period of time as in the present experiments. From the dilutions encountered it appears that the major pathway of utilization of adenine for nucleic acid biosynthesis in the rat does not involve its conversion to adenosine nor to the free adenylic acids *a* and *b* or AMP-5′.

The similar results with adenosine (17) and with the adenylic acids *a* and *b* permit the possibility that the nucleoside may have undergone phosphorylation to the *a* or *b* isomer or that the nucleotides may have been dephosphorylated to adenosine. Further experiments (19) with adenylic acids *a* and *b* labeled in the phosphate have revealed that the phosphate is not specifically utilized. It is quite probable that a major pathway of utilization of adenosine and of adenylic acids *a* and *b* for polynucleotide formation in the rat involves conversion to some other derivative of adenosine, but not necessarily direct transformation into AMP-5′.

With regard to formation of muscle adenosine polyphosphates, adenylic acids *a* and *b* produced appreciably greater renewals than did adenosine, in which case the value was 0.17 per cent for the ATP (17). Thus, while

the present results do admit the possibility that adenylic acids *a* and *b* may pass through adenosine prior to their utilization for polynucleotide synthesis, they suggest that adenosine may not be an intermediate in the conversion of these nucleotides to muscle ATP in the rat. (The large statistical error in the determination of the specific activity of the ATP adenine in the AMP-5′ experiment does not allow comparison of it with the result obtained in the adenosine experiment.)

In the previous experiments with N^{15}-labeled mixed guanylic acid isomers at twice the dose used here (3), the incorporation into PNA guanine was 2.2 per cent, or twice the amount found with guanylic acid *b*. A similarly satisfactory correlation of extent of incorporation with amount of compound administered is found with the mixed adenylic acids in which the incorporation into PNA adenine was 3.8 per cent when the nucleotides were administered at 0.4 mmole per kilo of body weight per day for 3 days (3). Although it was not practical to test this directly, it can thus be inferred that guanylic acid *a* is as effective a precursor of polynucleotide guanine as is guanylic acid *b*.

Guanylic acid *b* was about one-half as effective as a precursor of PNA guanine as were adenylic acids *a* and *b* of PNA adenine. As a source of PNA guanine, guanylic acid *b*-C^{14} was of the order of 4 and 10 times as effective as were guanosine-C^{14} (17) and guanine-C^{14} (15), respectively, thus confirming the unique position of guanylic acid (3).

There was a small conversion of the guanine of guanylic acid *b* to PNA adenine. This was not previously detected (3) when the less sensitive N^{15} was used as a tracer. Guanosine is one-tenth as active in this respect (17), and any conversion of guanine to adenine was not detectable (15). The present results do furnish some evidence that adenine and guanine derivatives are interconvertible in the rat in a restricted sense, since free base adenine or adenine in nucleoside or nucleotide linkage is converted to polynucleotide guanine, and guanine is converted to nucleic acid adenine to a small extent when furnished in nucleotide linkage. Abrams observed some conversion of free base guanine to polynucleotide adenine in rat intestine (20) and in rabbit bone marrow slices (21).

The purines of the PNA adenylic acids *a* and *b* were almost equally labeled, as were the purines of guanylic acids *a* and *b*, after the administration of each of the four nucleotides. Similar equal labeling of the pairs of isomers has been previously found with formate (22), adenine (16), and several nucleosides (17). The *a* and *b* isomers are obtained upon alkaline hydrolysis of PNA. If the mechanisms of their formation are valid,[3] equal labeling of the members of the isomeric pairs would be expected, and thus

[3] As proposed by Brown and Todd (23) and Lipkin, Talbert, and Cohn (24), complete randomization of PNA phosphorus between carbons 2 and 3 of ribose during the course of the hydrolysis is involved.

XUM

isolation of each isomer could be considered as a duplicate confirmatory measurement.

SUMMARY

Adenosine-2′-phosphate, adenosine-3′-phosphate, adenosine-5′-phosphate, and guanylic acid *b* (3′), labeled with C^{14} in the purine moieties, were administered to rats.

The adenosine-2′- and 3′-phosphates were utilized equally well as sources of polynucleotide adenine and guanine. As with the yeast adenylic acid-N^{15}, they were about as effective as adenosine, but less so than adenine. The 5′ isomer appeared to be about half as effective as the 2′ and 3′ isomers.

The 2′ and 3′ isomers of adenylic acid were more effective than AMP-5′ in labeling muscle adenosine polyphosphates.

The guanine of guanylic acid *b* (3′) was incorporated into polynucleotide guanine to an extent similar to that found with yeast guanylic acid-N^{15}. It was also converted to pentose nucleic acid adenine to a small extent.

The authors wish to thank Dr. M. Earl Balis for the adenine-8-C^{14} used, Dr. H. Christine Reilly for a culture of *Torulopsis utilis* and advice on its cultivation, and Misses Kay Markel and Elizabeth Ilief for competent technical assistance.

BIBLIOGRAPHY

1. Weinfeld, H., and Roll, P. M., *Federation Proc.*, **12,** 287 (1953).
2. Brown, G. B., Roll, P. M., and Weinfeld, H., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **2,** 385 (1952).
3. Roll, P. M., and Weliky, I., *J. Biol. Chem.*, **213,** 509 (1955).
4. Carter, C. E., *J. Am. Chem. Soc.*, **72,** 1466 (1950).
5. Cohn, W. E., *J. Am. Chem. Soc.*, **72,** 1471 (1950).
6. Khym, J. X., Doherty, D. G., Volkin, E., and Cohn, W. E., *J. Am. Chem. Soc.*, **75,** 1262 (1953).
7. Cavalieri, L. F., *J. Am. Chem. Soc.*, **75,** 5268 (1953).
8. Kerr, S. E., Seraidarian, K., and Brown, G. B., *J. Biol. Chem.*, **188,** 207 (1951).
9. Schultz, A. S., and Atkin, L., *Arch. Biochem.*, **14,** 369 (1947).
10. Volkin, E., and Cohn, W. E., in Glick, D., Methods of biochemical analysis, New York, **1,** 287 (1954).
11. Davoll, J., and Lowy, B. A., *J. Am. Chem. Soc.*, **73,** 1650 (1951).
12. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **193,** 481 (1951).
13. Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72,** 4273 (1950).
14. Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., *J. Biol. Chem.*, **172,** 469 (1948).
15. Balis, M. E., Marrian, D. H., and Brown, G. B., *J. Am. Chem. Soc.*, **73,** 3319 (1951).
16. Marrian, D. H., Spicer, V. L., Balis, M. E., and Brown, G. B., *J. Biol. Chem.*, **189,** 533 (1951).

17. Lowy, B. A., Davoll, J., and Brown, G. B., *J. Biol. Chem.*, **197**, 591 (1952).
18. LePage, G. A., *Biochem. Preparations*, **1**, 5 (1949).
19. Roll, P. M., Weinfeld, H., and Brown, G. B., *Biochim. et biophys. acta*, **13**, 141 (1954).
20. Abrams, R., *Arch. Biochem. and Biophys.*, **33**, 436 (1951).
21. Abrams, R., and Goldinger, J. M., *Arch. Biochem.*, **30**, 261 (1951).
22. Drochmans, P., Marrian, D. H., and Brown, G. B., *Arch. Biochem. and Biophys.*, **39**, 310 (1952).
23. Brown, D. M., and Todd, A. R., *J. Chem. Soc.*, 2040 (1953).
24. Lipkin, D., Talbert, P. T., and Cohn, M., *J. Am. Chem. Soc.*, **76**, 2871 (1954).

ENZYMATIC CARBON DIOXIDE FIXATION INTO OXALACETATE IN WHEAT GERM*

By T. T. TCHEN and BIRGIT VENNESLAND

(From the Department of Biochemistry, University of Chicago, Chicago, Illinois)

(Received for publication, August 9, 1954)

Bandurski and Greiner (1) have described an enzyme preparation from spinach leaves which catalyzes the irreversible addition of carbon dioxide to phosphoenolpyruvate to form oxalacetate and inorganic phosphate according to Reaction 1.[1] Reaction 1 differs from the reversible CO_2-

$$PEP + CO_2 + OH^- \rightarrow OAA + P_i \quad (1)$$

fixing reaction catalyzed by enzyme preparations from bird liver, which Utter and his collaborators (2–5) have shown to proceed according to Reaction 2. In the presence of an ADP-ITP transphosphorylating en-

$$PEP + CO_2 + IDP \rightleftharpoons OAA + ITP \quad (2)$$

zyme and a trace of ITP, the reaction appears to proceed according to Reaction 3.

$$PEP + CO_2 + ADP \rightleftharpoons OAA + ATP \quad (3)$$

The present investigation concerns primarily a study of the CO_2-fixing reactions catalyzed by enzyme preparations from wheat germ. This paper describes the evidence that wheat germ extracts catalyze Reactions 1, 2, and 3. The characteristics of Reaction 1 were studied in most detail.

Materials and Methods

The Ba salt of 3-PGA (Nutritional Biochemicals Corporation) was dissolved in dilute HCl and the Ba was precipitated by addition of a slight excess of Na_2SO_4. The supernatant liquid obtained on centrifugation

* Supported in part by grants from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. Part of the material in this paper is taken from a thesis submitted by T. T. Tchen in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[1] The following abbreviations are used: OAA, oxalacetate or oxalacetic acid; PEP, phosphoenolpyruvate; 2-PGA and 3-PGA, 2- and 3-phosphoglyceric acid, respectively; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; and AMP, adenosinemonophosphate; ITP, inosinetriphosphate; IDP, inosinediphosphate; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane.

XUM

was neutralized with solid $NaHCO_3$. The Ag-Ba double salt of PEP was synthesized according to Ohlmeyer (6) (80 per cent purity). This material was dissolved in dilute HNO_3, and the Ag^+ and Ba^{++} were precipitated by a slight excess of HCl and H_2SO_4. The supernatant liquid obtained by centrifugation was neutralized with solid $NaHCO_3$.

Inorganic phosphate was determined by the isobutanol-benzene extraction method of Ernster *et al.* (7). PEP was determined as mercury-labile phosphate (8). 1 volume of 10 per cent trichloroacetic acid was added to the reaction mixture, and the protein was removed by centrifugation. Two 1 ml. aliquots of the supernatant fluid (containing not more than 20 γ of mercury-labile phosphate plus inorganic phosphate) were measured out, and 0.1 ml. of a solution of 5 per cent $Hg(NO_3)_2$ was added to one of the aliquots. After 10 minutes at room temperature, 1 ml. of saturated K_2SO_4 was added and the volume was made up to 3 ml. with water. The inorganic phosphate was extracted with benzene-isobutanol. The difference in inorganic phosphate extracted with and without mercury treatment represents the amount of PEP in the solution.

ATP was the disodium salt (Pabst) and AMP was the free acid (Pabst). Solutions of the latter were neutralized before use. The Ba salt of ADP (Schwarz) and the Ba salt of ITP (Sigma) were dissolved in a minimal amount of HCl. The Ba was precipitated with an equivalent amount of Na_2SO_4 and the precipitate was removed by centrifugation. The supernatant fluid was adjusted to neutral pH with solid Na_2CO_3.

Enzyme—Wheat germ (General Mills, S-50) was extracted with 4 volumes of water for 30 minutes at room temperature. The solids were removed by centrifugation. Preparation WH was dialyzed at 4° against distilled water. Preparation WC was dialyzed at 4° against 0.01 M collidine buffer of pH 6.8. Preparation WMH was treated with 20 ml. of 1 M $MnCl_2$ per liter of extract. The precipitate was removed by centrifugation and the supernatant fluid was dialyzed at 4° against distilled water. Preparation WMC was identical with Preparation WMH, except that the final dialysis was carried out against 0.01 M collidine of pH 6.8. All these enzyme preparations contained phosphoglycerate mutase, enolase, malic dehydrogenase (9), and alcohol dehydrogenase (10), but no lactic dehydrogenase and no fumarase.

Collidine (2,4,6-trimethylpyridine) was obtained from The Matheson Company. The fraction boiling in the range 169.5–171° (uncorrected) was used. The pH of buffer solutions was adjusted with HCl. Insoluble material was allowed to settle out and was discarded. Other materials and methods are described in a preceding paper (9). All experiments were done at room temperature.

EXPERIMENTAL

Oxidation of DPNH by PEP and 3-PGA—Both PEP and 3-PGA cause an oxidation of DPNH in the presence of dialyzed wheat germ extracts. This reaction can be followed spectrophotometrically by measurement of the light absorption at 340 mμ. The results of a typical experiment are presented in Fig. 1. In the absence of added oxidant, there is a slow disappearance of DPNH. When PEP is added, the DPNH is oxidized

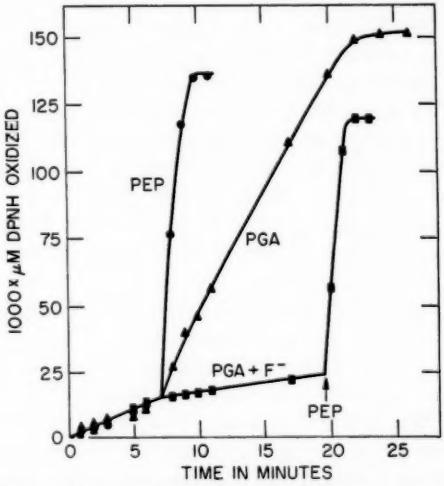


FIG. 1. Oxidation of DPNH by PEP and PGA. The reaction mixtures contained 0.02 M phosphate buffer, pH 7.0, 0.003 M $MgCl_2$, 0.01 M NaF as indicated, 2 μmoles of PEP or 3-PGA, and 0.1 ml. of wheat germ Preparation WMH in a total volume of 3 ml. In all these experiments, there was sufficient bicarbonate in the reagents to allow the reaction to proceed at maximal velocity.

rapidly and completely. The DPNH is also reoxidized completely by 3-PGA, but at a slower rate. Furthermore, the reaction with 3-PGA is inhibited by fluoride, whereas the reaction with PEP is not. The enzyme reaction mixture in these experiments contained phosphate and Mg^{++}. The results, therefore, suggest that the fluoride is inhibiting enolase, and that 3-PGA is converted to PEP, which is the more immediate oxidant of DPNH.

Identification of Malate—The reduction of PEP by DPNH involved a fixation of CO_2. The product of the reaction was identified as malate. By running the reaction in the presence of $C^{14}O_2$, it was also shown that

the β-carboxyl group of the malate was derived from CO_2. In a typical experiment, the reaction mixture contained 50 mg. of the Tris salt of DPNH, 14.2 μmoles of PEP, and 1.2 ml. of wheat germ Preparation WMH in a total volume of 12 ml. of 0.02 M collidine buffer of pH 6.8, containing 0.01 M $MgCl_2$ and $NaHC^{14}O_3$. The mixture was incubated at room temperature for 60 minutes, at the end of which time the reaction was complete. The mixture was then acidified, deproteinized, and extracted with ether, and the extract was analyzed for malate. These operations were carried out as described elsewhere (9). From the 14.2 μmoles of PEP added, 12.6 μmoles of malate were obtained, amounting to 89 per cent of the PEP added. During the reaction, 0.7 μmole of pyruvate was also formed, as determined by the method of Lu (11).

Determinations of the C^{14} activity of the various fractions showed that C^{14} was fixed in organic form during the incubation. All the detectable C^{14} fixed was extracted into ether. A sample was chromatographed on paper according to Lugg and Overell (12). All the radioactivity, amounting to 16,200 c.p.m., appeared with the malic acid. When the malate was decarboxylated with *Lactobacillus arabinosus* (13), 98 per cent of the fixed C^{14} was removed as CO_2.

Evidence for Two-Step Reaction—The formation of malate from PEP and CO_2 was shown to occur in two separate steps. The first step consists of an addition of CO_2 to PEP to form oxalacetate, as shown in Reaction 1. The second step is a reduction of the oxalacetate to malate by DPNH (Reaction 4). This reaction is catalyzed by malic dehydrogenase, which is very active in the wheat germ preparation. Reaction 4 is so rapid, relative to Reaction 1, that the rate of the over-all Reaction 5 is practically identical to the rate of Reaction 1.

$$OAA + DPNH + H^+ \rightleftharpoons \text{malate} + DPN^+ \quad (4)$$

$$PEP + CO_2 + DPNH \rightarrow \text{malate} + DPN^+ + P_i \quad (5)$$

It was possible to demonstrate the separate occurrence, in sequence, of Reactions 1 and 4 with the same enzyme preparation. This was done in the following way. PEP was first incubated with the enzyme in the absence of DPNH. As shown in a later section, the rate of Reaction 1 is not influenced by the removal of OAA. Aliquots of the reaction mixture taken at successive time intervals contained progressively increasing amounts of OAA. If such aliquots are added to samples of DPNH strongly buffered with acetate at pH 5.4, no further OAA is formed, since Reaction 1 is inhibited at this pH. Since the malic dehydrogenase is still active at pH 5.4, the OAA already formed oxidizes an equivalent amount of DPNH. This reaction is complete under the conditions used, within less than 2 minutes. During this time the blank rate of disappearance of DPNH

is negligible. Fig. 2 shows a plot against time of the accumulation of OAA, determined as just described. One curve shows the results obtained when the initial incubation was carried out in phosphate buffer; the other curve shows the results obtained under otherwise identical conditions when collidine buffer was substituted for phosphate. More OAA was formed in phosphate than in collidine.

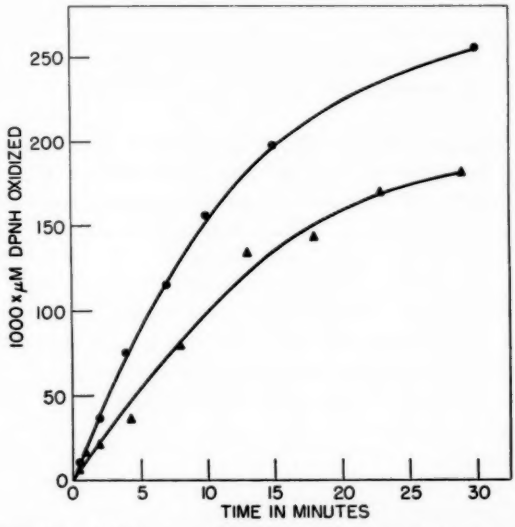


FIG. 2. Formation of OAA from PEP. The reaction mixtures contained 0.02 M phosphate (●) or collidine buffer (▲), pH 7.0, 0.01 M $MgCl_2$, 10 μmoles of PEP, and 0.4 ml. of wheat germ Preparation WMH in a total volume of 2 ml. At the indicated time intervals, 0.2 ml. aliquots were pipetted into cuvettes containing 2.8 ml. of 0.03 M acetate buffer, pH 5.4, and excess DPNH. The plot shows the total amount of DPNH oxidized in each sample.

The OAA formed in the absence of DPNH by Reaction 1 was also identified by the preparation of its 2,4-dinitrophenylhydrazone. It was also shown that 3-PGA yielded the same products as PEP, that OAA is formed from 3-PGA in the absence of DPNH, and that this OAA is reduced quantitatively to malate when DPNH is added. These conclusions are supported by the following experiment. A reaction mixture was made up to contain 60 μmoles of phosphate buffer of pH 7.0, 30 μmoles of $MgCl_2$, 25 μmoles of 3-PGA, $NaHC^{14}O_3$, and 0.5 ml. of wheat germ Preparation WMH in a total volume of 2.5 ml. The mixture was incubated for 17 minutes at room temperature. Two 1 ml. samples were then pipetted,

respectively, into each of two tubes. One tube contained 42 mg. of carrier OAA dissolved in 2 N HCl; the other tube contained 2.5 μmoles of DPNH in 2 ml. of 0.05 M acetate buffer of pH 5.4.

From the first tube, the protein was precipitated by adding 0.1 volume of 20 per cent metaphosphoric acid, and the 2,4-dinitrophenylhydrazone of OAA was prepared and recrystallized to constant radioactivity, essentially as described by Bandurski and Greiner (1). From the other tube, malate was extracted with ether. The total radioactivity in the malate and in the OAA, after correction for yield, was 4580 and 4481 c.p.m., respectively. The malate was separated and identified by paper chromatography and by decarboxylation with *L. arabinosus*, as in the previous experiment.

Phosphate Changes—When PEP is converted to OAA in the presence of wheat germ, inorganic phosphate appears (Reaction 1). Experiments showed that the rate of inorganic phosphate formation is the same, whether DPNH is present or absent. The changes with time of mercury-labile and inorganic phosphate are shown in Fig. 3. At the beginning of the reaction, the rate of disappearance of PEP is greater than the rate of appearance of inorganic phosphate. A stable phosphate ester (labeled *X* on the figure) accumulates and then disappears. All of the phosphate of PEP is eventually converted to inorganic phosphate. These results are compatible with the fact that the enzyme preparation contains enolase and phosphoglyceromutase.

At the beginning of the incubation, PEP is removed by two different reaction paths, *i.e.* by the formation of 2-PGA and 3-PGA on the one hand, and the formation of OAA and inorganic phosphate on the other. As the concentration of PEP decreases, the 2- and 3-PGA are reconverted to PEP which is eventually completely converted to OAA and inorganic phosphate. When these reactions occur in the presence of DPNH, the rate of oxidation of DPNH was found to be equal to the rate of formation of inorganic phosphate, showing again that Reaction 1 is the rate-limiting step in the over-all Reaction 5.

Cation Requirement—The formation of OAA from PEP is stimulated by Mg^{++} ions. This was conveniently demonstrated by showing the effect of Mg^{++} on the rate of DPNH oxidation in the presence of PEP, as measured in the spectrophotometer. Such an experiment is shown in Fig. 4. The Mg^{++} must be affecting the rate of OAA formation, since the oxidation of DPNH by OAA is not stimulated by Mg^{++}. (An exception to this statement has been described in another paper (9), but it does not apply here (14).) Even after extensive dialysis, no absolute requirement for Mg^{++} has been obtained. However, 3.3×10^{-5} M Versene inhibits the reaction completely in the absence of added Mg^{++}. Addition of an excess

of $MgCl_2$ (final concentration, 0.01 M) reverses this inhibition completely. It was not possible to use the spectrophotometric test to determine the effect of Mn^{++} on Reaction 1 because Mn^{++} causes an oxidation of DPNH. When Reaction 1 was followed by measuring the rate of liberation of inorganic phosphate, 0.01 M $MnCl_2$ was found to stimulate the reaction to the same extent as 0.01 M $MgCl_2$. The spectrophotometric test was

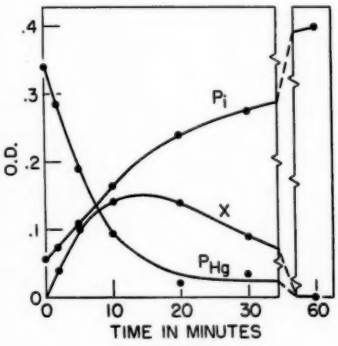


FIG. 3

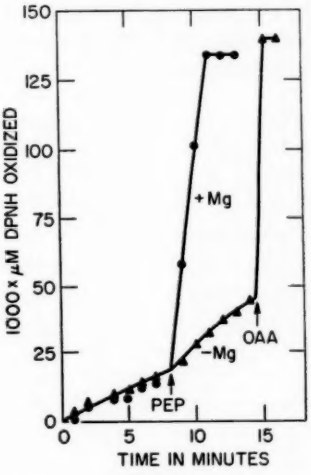


FIG. 4

FIG. 3. Fate of phosphate. The reaction mixtures contained 0.02 M collidine buffer, pH 7.0, 0.01 M $MgCl_2$, 5.3 μmoles of PEP, and 1.5 ml. of wheat germ Preparation WMH in a total volume of 8 ml. At the indicated time intervals, 1 ml. aliquots were removed for phosphate analyses. A change of 0.100 in optical density corresponds to 6 γ of phosphorus in the 1 ml. aliquot.

FIG. 4. Effect of Mg^{++}. The reaction mixtures contained 0.02 M phosphate buffer, pH 7.0, 0.01 M $MgCl_2$, as indicated, 2 μmoles of PEP, and 0.1 ml. of wheat germ Preparation WMH in a total volume of 3.0 ml.

also used to examine the possible effects of Na^+ and K^+. The medium was made up to contain Na^+ as the only monovalent inorganic cation in one case and K^+ as the only monovalent inorganic cation in the other. The rate of the reaction was the same, whether the medium contained Na^+ or K^+.

Effect of SH Reagents—The CO_2-fixing reaction, measured spectrophotometrically, was not stimulated by treatment of the enzyme with reduced glutathione. This is contrary to the results of Bandurski and Greiner (1), who reported that treatment with such a reducing agent was required in order to obtain an active enzyme from spinach leaves. A spinach

enzyme prepared according to the directions of Axelrod *et al.* (15) behaved in this respect like the wheat germ; *i.e.*, pretreatment with cysteine or reduced glutathione was neither required for activation of the enzyme nor did it have a stimulatory effect.

The ability of wheat germ to catalyze Reaction 5 was not inhibited by 0.01 M iodoacetate, but was completely inhibited by 1×10^{-5} M *p*-chloromercuribenzoate, as shown in Fig. 5. Under these conditions, the malic dehydrogenase was still active, as shown by the fact that added OAA was rapidly reduced. The *p*-chloromercuribenzoate must therefore have inhibited Reaction 1.

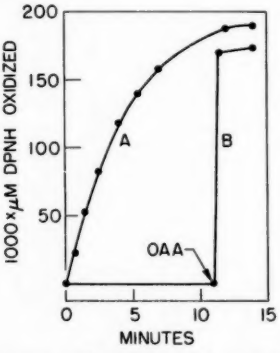


FIG. 5. Effect of *p*-chloromercuribenzoate. The reaction mixtures contained 0.02 M phosphate buffer, pH 7.0, 0.01 M $MgCl_2$, 2 μmoles of PEP, and 0.1 ml. of wheat germ Preparation WMH in a total volume of 3.0 ml. Curve A, no *p*-chloromercuribenzoate; Curve B, the reaction mixture contained 10^{-5} M *p*-chloromercuribenzoate.

Effect of Nucleotides and Phosphate—ATP, ADP, and AMP at low concentrations had no effect on the spectrophotometric reaction. At a concentration of 3.3×10^{-3} M, small inhibitory effects were noted. The conditions used were those described in the legend for Fig. 1.

The rate of OAA formation was stimulated by inorganic phosphate. The results, already described in Fig. 2, showed that OAA was formed more rapidly in phosphate buffer than in collidine buffer of the same pH and concentration. The rate of OAA formation measured spectrophotometrically, as in Fig. 1, was also greater when the reaction was carried out in phosphate buffer than when it was carried out in collidine buffer. This was shown to be due to a true stimulatory effect by phosphate and not to any inhibition by collidine, since addition of small amounts of phosphate to a reaction mixture buffered with collidine stimulated the reaction,

whereas addition of collidine to a reaction mixture buffered with phosphate caused no change in reaction rate.

The phosphate stimulation was not completely reproducible quantitatively, but varied with the freshness of the enzyme preparation and of the PEP solution. It is difficult to free the reaction mixture of inorganic phosphate. Account must also be taken of the fact that inorganic phosphate is formed during Reaction 1. The lowest concentration of phosphate at which a small but definite stimulation was consistently observed was 0.1 μmole per 3 ml. At a concentration of 8 μmoles per 3 ml., the reaction rate was usually approximately doubled. Arsenate at this concentration also stimulated the reaction to a comparable extent.

Since inorganic phosphate stimulated OAA formation at such a low concentration, a possible function of inorganic phosphate in Reaction 1 was also investigated. It appeared, for example, that inorganic phosphate might act as a phosphate acceptor in the same way that IDP functions in Reaction 2. Under such circumstances, pyrophosphate would be formed. The pyrophosphate would be hydrolyzed, however, by the pyrophosphatase present in wheat germ. PEP, P_i^{32}, and an excess of pyrophosphate were therefore incubated with the enzyme and the residual pyrophosphate was isolated according to the procedure described by Kornberg (16). No radioactivity was found in the pyrophosphate, indicating that inorganic phosphate does not act as a phosphate acceptor. In another experiment, an excess of PEP was incubated with P_i^{32} and enzyme. The solution was deproteinized and decationized with Dowex 50, and then chromatographed, according to Bandurski and Axelrod (17). Only the inorganic phosphate spot contained radioactivity. This indicated that there is no exchange between inorganic phosphate and the phosphate moiety of PEP. It also showed the irreversibility of Reaction 1.

Reaction 1 in Spinach—Except for the experiments with iodoacetate, all the various experiments described in the preceding sections were also carried out with a spinach preparation similar to that used by Bandurski and Greiner (1). The results were essentially identical to those obtained with wheat germ. A trivial qualitative difference appeared only in the finding that the reaction catalyzed by spinach enzyme shows a more marked requirement for Mg^{++}. Except for minor differences these observations confirm and extend the conclusions of the California workers regarding the occurrence and the properties of Reaction 1 in spinach.

ATP-Dependent Exchange Reaction—The exchange of $C^{14}O_2$ into the β-carboxyl group of OAA (Reaction 6) was determined by incubating OAA and $C^{14}O_2$ with wheat germ under a variety of conditions. The reaction

$$COO^-\cdot CO\cdot CH_2\cdot COO^- + C^{14}O_2 \rightarrow COO^-\cdot CO\cdot CH_2\cdot C^{14}OO^- + CO_2 \qquad (6)$$

was stopped by addition of citric acid, the $C^{14}O_2$ was swept out with unlabeled CO_2, the oxalacetate was decarboxylated with aniline citrate, and the $C^{14}O_2$ was collected for radioactivity determination. The details of the procedure have been described elsewhere (18).

In the presence of added ATP or ITP, wheat germ Preparations WC and WH catalyzed on active exchange reaction. The dependence of this reaction on ATP is shown in Fig. 6. ITP, tested at a concentration of

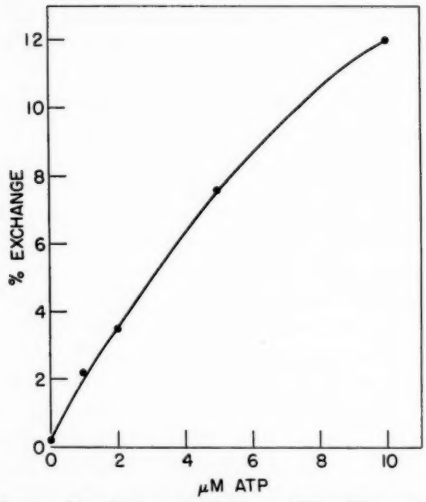


FIG. 6. Dependence of exchange reaction on ATP. The reaction mixtures contained 0.02 M collidine buffer, pH 7.0, 0.01 M $MnCl_2$, 10 μmoles of OAA, and 1 ml. of wheat germ Preparation WC in a total volume of 10 ml. The results are expressed as the per cent of the β-carboxyl carbon of OAA which is derived from the total CO_2 and bicarbonate of the medium.

2×10^{-3} M, is as effective in stimulating the exchange reaction as an equal concentration of ATP.

The reaction is also strongly stimulated by the addition of Mg^{++} or Mn^{++} ions. $MgCl_2$ and $MnCl_2$ are equally effective at a concentration of 0.01 M. The exchange observed in the absence of divalent cations varied from preparation to preparation, but was always low. In a typical experiment the per cent exchange dropped from 10.2 per cent with 0.01 M $MnCl_2$ to 1.5 per cent without added $MnCl_2$. The conditions of this experiment were the same as those for Fig. 6.

It was possible to demonstrate experimentally that the exchange reaction of wheat germ was not catalyzed by the same enzyme (or enzymes) that

catalyzed the irreversible formation of oxalacetate and phosphate from PEP and CO_2. Thus, the wheat germ extracts lost about 75 per cent of the exchange reaction activity on treatment of the crude preparation with $MnCl_2$ as described in the section on preparation of the enzyme. This treatment had no effect on the ability of the extracts to catalyze Reaction 1. Even more striking was the effect of dialysis against collidine buffer. This treatment caused complete inactivation of the enzyme catalyzing Reaction 1, but left intact the catalytic effect on the exchange reaction.

It is not clear why dialysis against collidine buffer should inactivate the enzyme for Reaction 1, although dialysis against water has no inactivating effect. The preparation inactivated by dialysis against collidine buffer could not be reactivated by addition of inorganic phosphate. Addition of 0.01 M collidine buffer to the reaction mixture in itself caused no inactivation of the enzyme catalyzing Reaction 1, even on prolonged standing.

Since Reaction 1 can occur in the absence of the exchange reaction, the latter must indicate the presence of a different or independent enzyme system. The exchange reaction is regarded as good presumptive evidence for the occurrence of Reactions 2 and 3, since these reactions would account completely for the observed properties of the system.

DISCUSSION

The mechanism of formation of oxalacetate from PEP and CO_2 is the subject of another paper (14). The discussion here may be limited to the question of physiological function. Utter and Kurahashi (5) have already pointed out the possible significance of Reactions 2 and 3 in the formation of carbohydrate. These reactions provide a means of synthesizing appreciable amounts of PEP from OAA and either ATP or ITP, and thus explain the older observation of Kalckar (19) that PEP accumulates during the oxidation of the Krebs cycle intermediates by preparations of particulates. Similar observations have also been made by Leloir and Muñoz (20), by Neuberg and Mudge (21), and by Bartley (22).

Krebs[2] has concluded, on the basis of rate measurements, that the three reactions (*a*, *b*, and *c*) are sufficiently rapid in animal tissues to account

$$\text{Pyruvate} + CO_2 + \text{TPNH} \rightleftharpoons \text{malate} + \text{TPN}^+ \qquad (a)$$

$$\text{Malate} + \text{DPN}^+ \rightleftharpoons \text{OAA} + \text{DPNH} + H^+ \qquad (b)$$

$$\text{OAA} + \text{ATP} \rightleftharpoons \text{PEP} + CO_2 + \text{ADP} \qquad (c)$$

$$\text{Pyruvate} + \text{ATP} \rightleftharpoons \text{PEP} + \text{ADP} \qquad (d)$$

(together with the other necessary reactions) for the synthesis of PEP

[2] H. A. Krebs, material presented in a lecture at Chicago, March, 1954.

from pyruvate, a step which is essential for the conversion of pyruvate to carbohydrate. Reaction *d*, on the other hand, is far too slow to form PEP at the required rate. We are in agreement with Utter and Kurahashi, and with Krebs, in regard to the conclusion that the reversible carboxylation of PEP in the presence of ADP to form OAA and ATP is probably a key reaction in the formation of PEP, and consequently of carbohydrate, from the precursors which form part of the citric acid cycle. Since this point has already been discussed in detail, it will not be elaborated here. It seems appropriate to indicate, however, that wheat germ is a good source of the enzymes catalyzing Reactions *a* and *b* (23), and, if the indirect evidence presented in this paper for the occurrence of Reaction *c* is valid, then it would appear that wheat germ can form PEP from pyruvate by this route, just as do animal tissues. The occurrence of Reactions 2 and 3 in leaves has not yet been reported. It will be interesting to see whether these reactions occur in photosynthesizing tissue in which carbohydrate is synthesized from PGA which arises by a photosynthetic CO_2 fixation reaction not involving PEP (24, 25).

Utter and Kurahashi (3) have pointed out that Reaction *c* may be difficult or impossible to demonstrate directly in the presence of pyruvic phosphokinase catalyzing Reaction *d*. Under these circumstances, the ATP- or ITP-dependent exchange reaction may provide the best presumptive evidence for the enzyme catalyzing the reversible CO_2-fixing reaction.

The irreversible formation of OAA from PEP and CO_2 according to Reaction 1 has hitherto been demonstrated only in spinach leaves and in wheat germ. It may have a wide distribution in plants, although this has not yet been reported, but it has not yet been demonstrated in animal tissues. Utter and Kurahashi (5) found no evidence that such a reaction occurred in the presence of their purified liver enzyme which catalyzes Reaction 2. This is in agreement with the conclusion reached in the present paper that the irreversible and the reversible CO_2-fixing reactions of wheat germ are catalyzed by different enzymes or enzyme systems. In spite of the separability of these reactions in wheat germ, it is not impossible that they may contain a common step. This question is discussed elsewhere (14).

Since Reaction 1 is irreversible, it clearly cannot function in the formation of PEP. This enzyme actually removes PEP very efficiently from a reaction mixture, provided CO_2 is available. Similarly, OAA is formed at very low CO_2 tensions, provided PEP is present. In fact, it was not possible to demonstrate a CO_2 requirement for the reaction by use of the routine spectrophotometric method described in this paper. Precautions were taken to remove CO_2 from all reagents, but the lyophilized enzyme preparation apparently still contained enough bicarbonate to allow the

reaction to proceed at an undiminished rate. The participation of CO_2 in the reaction could only be demonstrated readily with isotopic carbon. The reversible OAA-forming reaction and the malic enzyme reaction (26) both require a relatively high concentration of CO_2 to form an appreciable amount of dicarboxylic acid. Reaction 1 seems peculiarly suited, therefore, for the effective conversion of carbohydrate to dicarboxylic acids at low CO_2 tensions.

It is known that some plants accumulate organic acids, mainly malic acid, during the night. The subject has been reviewed extensively and it is generally agreed that malate is formed from carbohydrate by way of CO_2 addition to a C_3 unit (27–31). It has also been shown that some acid accumulation occurs at an extremely low CO_2 tension. Although an increase in CO_2 tension causes an increase in acid formation, there is an appreciable amount of acid formed even in CO_2-free air (32). It is possible that Reaction 1, the irreversible formation of OAA from PEP and CO_2, is the key reaction in the dark accumulation of malic acid in plants when the CO_2 pressure is low. The accumulation of malic acid in the dark might be regarded as a means of storing the CO_2 produced by respiration, and the irreversible formation of OAA from PEP may thus function as a very efficient trap for CO_2. This does not imply that the reversible CO_2-fixing systems are not concerned in the acid formation. The reversible reactions, however, require higher CO_2 tensions in order to form appreciable quantities of acid. Further investigation of the distribution of the various enzymes and the rates of the various reactions is required in order to assess the relative importance of the different CO_2-fixing systems.

SUMMARY

Evidence is presented that wheat germ extracts cause the enzymatic formation of OAA from PEP and CO_2 by two different reactions. One of these is reversible and requires ADP or IDP as a phosphate acceptor. The other reaction is irreversible and involves a liberation of inorganic phosphate. The properties of the second reaction have been investigated, and the physiological significance of the reactions has been discussed.

We are indebted to the research department of General Mills, Inc., of Minneapolis for a generous sample of wheat germ S-50.

BIBLIOGRAPHY

1. Bandurski, R. S., and Greiner, C. M., *J. Biol. Chem.*, **204**, 781 (1953).
2. Utter, M. F., and Kurahashi, K., *J. Am. Chem. Soc.*, **75**, 758 (1953).
3. Utter, M. F., and Kurahashi, K., *J. Biol. Chem.*, **207**, 787 (1954).
4. Utter, M. F., Kurahashi, K., and Rose, I. A., *J. Biol. Chem.*, **207**, 803 (1954).
5. Utter, M. F., and Kurahashi, K., *J. Biol. Chem.*, **207**, 821 (1954).

6. Ohlmeyer, P., *J. Biol. Chem.*, **190,** 21 (1951).
7. Ernster, L., Zetterström, R., and Lindberg, R., *Acta chem. Scand.*, **4,** 942 (1950).
8. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273,** 60 (1934).
9. Loewus, F. A., Tchen, T. T., and Vennesland, B., *J. Biol. Chem.*, **212,** 787 (1955).
10. Stafford, H. A., and Vennesland, B., *Arch. Biochem. and Biophys.*, **44,** 404 (1953).
11. Lu, G. D., *Biochem. J.*, **33,** 249 (1939).
12. Lugg, W. H., and Overell, B. T., *Nature*, **160,** 87 (1947).
13. Korkes, S., del Campillo, A., and Ochoa, S., *J. Biol. Chem.*, **187,** 891 (1950).
14. Tchen, T. T., Loewus, F. A., and Vennesland, B., *J. Biol. Chem.*, **213,** 547 (1955).
15. Axelrod, B., Bandurski, R. S., Greiner, C. M., and Jang, R., *J. Biol. Chem.*, **202,** 619 (1953).
16. Kornberg, A., *J. Biol. Chem.*, **182,** 779 (1950).
17. Bandurski, R. S., and Axelrod, B., *J. Biol. Chem.*, **193,** 405 (1951).
18. Vennesland, B., Evans, E. A., Jr., and Altman, K. I., *J. Biol. Chem.*, **171,** 675 (1947).
19. Kalckar, H., *Biochem. J.*, **33,** 631 (1939).
20. Leloir, L. F., and Muñoz, J. M., *J. Biol. Chem.*, **153,** 53 (1944).
21. Neuberg, H. W., and Mudge, G. H., *Federation Proc.*, **13,** 104 (1954).
22. Bartley, W., *Biochem. J.*, **56,** 387 (1954).
23. Kraemer, L. M., Conn, E. E., and Vennesland, B., *J. Biol. Chem.*, **188,** 583 (1951).
24. Fager, E. W., Rosenberg, J. L., and Gaffron, H., *Federation Proc.*, **9,** 535 (1950).
25. Fager, E. W., *Arch. Biochem. and Biophys.*, **41,** 383 (1952).
26. Ochoa, S., in Sumner, J. B., and Myrbäck, K., The enzymes, New York, **2,** pt. 2, 929 (1952).
27. Bennet-Clark, T. A., *Ann. Rev. Biochem.*, **18,** 639 (1949).
28. Thimann, K. V., and Bonner, W. D., Jr., *Ann. Rev. Plant Physiol.*, **1,** 75 (1950).
29. Burris, R. H., *Ann. Rev. Plant Physiol.*, **4,** 91 (1953).
30. Thomas, H., Plant physiology, London (1947).
31. Vennesland, B., and Conn, E. E., *Ann. Rev. Plant Physiol.*, **3,** 307 (1952).
32. Bonner, W., and Bonner, J., *Am. J. Bot.*, **35,** 113 (1948).

THE MECHANISM OF ENZYMATIC CARBON DIOXIDE FIXATION INTO OXALACETATE*

By T. T. TCHEN, FRANK A. LOEWUS, AND BIRGIT VENNESLAND

(From the Department of Biochemistry, University of Chicago, Chicago, Illinois)

(Received for publication, August 9, 1954)

Studies of the non-enzymatic decarboxylation of β-keto acids have shown that it is the keto form of the β-keto acid which loses CO_2 to form an enol of the product. This was demonstrated for dimethylacetoacetic acid by Pedersen (1) and for dimethyloxalacetic acid by Steinberger and Westheimer (2). These latter authors studied the catalytic effect of metal ions on the decarboxylation reaction and suggested that the non-enzymatic reaction be taken as a model for similar enzymatic decarboxylations.

Kornberg *et al.* (3) observed the formation of an enol band during the enzymatic decarboxylation of oxalacetic acid and of another β-keto acid, oxalsuccinic acid. They regarded their results as evidence that an enol form of the β-keto acid was decarboxylated, although their data do not appear to preclude the possibility that an enol appears as a product of the decarboxylation (4).

Recent studies in this laboratory have shown that wheat germ extracts catalyze an irreversible carboxylation reaction which is essentially the reverse of a decarboxylation. This reaction consists of the addition of CO_2 to phosphoenolpyruvate to form oxalacetate and inorganic phosphate (5).[1] If the mechanism for the non-enzymatic decarboxylation should apply in reverse to this reaction, then the enol, PEP, should add CO_2 to give the keto form of oxalacetate. Experiments with deuterium described in this paper have provided unequivocal evidence that the oxalacetate formed enzymatically in the carboxylation reaction is in fact the keto form and not an enol. These results have been described in a preliminary publication (6).

* Supported in part by a grant from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, by research grant No. G-3222 from the National Institutes of Health, United States Public Health Service, and by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. Part of the material in this paper is taken from a thesis submitted by T. T. Tchen in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[1] The following abbreviations will be used: PEP, phosphoenolpyruvic acid; 2-PGA and 3-PGA, 2- and 3-phosphoglyceric acid, respectively; OAA, oxalacetate or oxalacetic acid; P_i, inorganic phosphate; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; ADP, adenosinediphosphate; ATP, adenosinetriphosphate; IDP, inosinediphosphate; ITP, inosinetriphosphate.

EXPERIMENTAL

The materials and most of the experimental procedures have been described elsewhere (5, 7). In Experiment 1, the reaction mixture contained 200 μmoles of phosphate buffer of pH 7.0, 150 μmoles of 3-PGA, 70 μmoles of $MgCl_2$, 5 μmoles of DPN^+, 1.0 ml. of unlabeled ethanol, 1 γ of crystalline yeast alcohol dehydrogenase (8), and 1.5 ml. of wheat germ Preparation WMH (5) in a total volume of 20 ml. of 82 per cent D_2O. The mixture was incubated at room temperature for 50 minutes. The reaction was stopped by addition of H_2SO_4, protein was precipitated by tungstate, and the malic acid was extracted with ether, analyzed, diluted with unlabeled malic acid, and converted to the diphenacyl derivative for deuterium analysis as previously described (7).

In Experiment 2, the reaction mixture contained 130 μmoles of 3-PGA, 150 μmoles of $MgCl_2$, and 2.0 ml. of enzyme, and the incubation was continued for 60 minutes. The other components of the reaction mixture and the procedure were identical with that for Experiment 1. In Experiment 3, the reaction mixture contained 200 μmoles of phosphate buffer of pH 7.0, 250 μmoles of 3-PGA, 50 μmoles of $MgCl_2$, 5 μmoles of DPN^+, 1.0 ml. of CH_3CD_2OH (9), and 1.5 ml. of wheat germ Preparation WMH in a total volume of 15 ml. of H_2O. The remainder of the procedure was identical with that described for Experiment 1.

RESULTS AND DISCUSSION

Evidence for Formation of Keto-OAA—Experiments described elsewhere (5, 7, 10) have shown that wheat germ contains all the enzymes necessary to catalyze the over-all reaction

$$\text{3-PGA} + CO_2 + \text{ethanol} \xrightarrow{Mg^{++},\ DPN} \text{malate} + \text{acetaldehyde} \qquad (1)$$

This over-all reaction represents the result of five separate enzymatic steps (*a*, *b*, *c*, *d*, and *e*). The third step, Reaction 1, *c*, is the one under investi-

$$\text{3-PGA} \rightleftharpoons \text{2-PGA} \qquad (1, a)$$

$$\text{2-PGA} \rightleftharpoons \text{PEP} + H_2O \qquad (1, b)$$

$$\text{PEP} + CO_2 + OH^- \rightarrow \text{OAA} + P_i \qquad (1, c)$$

$$\text{OAA} + \text{DPNH} \rightleftharpoons \text{malate} + DPN^+ + H^+ \qquad (1, d)$$

$$\text{Ethanol} + DPN^+ \rightleftharpoons \text{acetaldehyde} + \text{DPNH} + H^+ \qquad (1, e)$$

gation. The nature of the OAA formed in this step was determined by running the over-all reaction in a medium of D_2O, isolating the malate as the diphenacyl derivative, and determining the amount of non-exchangeable deuterium present in the malate.

Consideration of the properties of each of the separate steps shows that the only reasonable route by which D can be introduced into malate in a non-exchangeable position is by way of an enol form of OAA. Thus, the known structural formulas and properties of 3-PGA, 2-PGA, and PEP show that no D from the medium should be incorporated into the methylene group of PEP during the enzymatic equilibration of these compounds by Reactions 1, *a* and 1, *b*. Reaction 1, *d*, catalyzed by malic dehydrogenase, has been shown to involve a reduction of the keto form of OAA to malate by direct transfer of H from DPNH to the carbonyl carbon atom (7). In Reaction 1, *e*, DPN^+ is reduced to DPNH by direct transfer of H from the α-carbon atom of ethanol. When Reactions 1, *d* and 1, *e* are

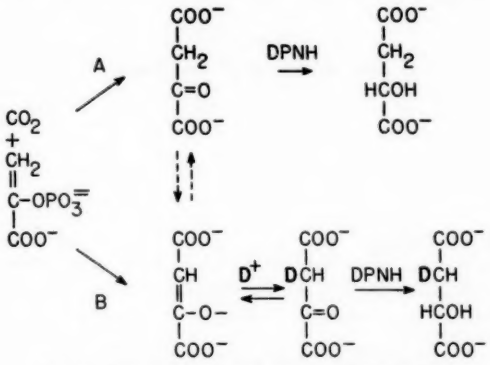


FIG. 1. Formation of malate from PEP in D_2O

coupled, the malate acquires H from ethanol by way of DPN, and no non-exchangeable D from the medium is introduced into malate unless the OAA which is reduced already contains D in the methylene group.

We are left, then, with two possibilities for introducing non-exchangeable D into malate. If the OAA formed in Reaction 1, *c* is an enol, a minimum of 1 atom of D must be introduced from the medium during the ketonization of OAA which must precede Reaction 1, *d*. This is shown in Path B of Fig. 1. If the reaction proceeds by Path A of Fig. 1, D can be introduced into the methylene group of OAA only by the spontaneous, non-enzymatic keto-enol tautomerization of OAA (broken arrows of Fig. 1). The quantity of D introduced into malate will then be a function of the relative rates of the enzymatic reduction of OAA and of the spontaneous tautomerization of OAA. If the reduction proceeds considerably more rapidly than the tautomerization, only small amounts of non-exchangeable D will be incorporated into malate. Such a result would provide evidence that the reaction occurs by Path A and not by Path B.

Although the tautomerization of OAA is rapid under the experimental conditions employed, the enzymatic reduction of OAA is also very rapid. Furthermore, it has been shown previously that Reaction 1, *c* proceeds much more slowly than Reaction 1, *d* in the wheat germ extracts; hence the OAA is reduced almost as rapidly as it is formed (5). The equilibrium of Reaction 1, *d* lies far to the right, and the reduction of OAA was further favored by using a large excess of ethanol for Reaction 1, *e*. Although wheat germ contains an active alcohol dehydrogenase, extra crystalline alcohol dehydrogenase from yeast was added to increase the velocity of Reaction 1, *e*.

Under these circumstances, the malate formed by the over-all Reaction 1 contained 0.10 atom of D per molecule in Experiment 1 and 0.05 atom

TABLE I

Incorporation of D into Malate

Experiment No.	Reductant	Medium	Malic acid		Atom per cent excess D*		Atoms D incorporated per molecule malate
			Formed	Added	Theoretical†	Found	
			mg.	*mg.*			
1	$DPN^+ + CH_3CH_2OH$	82% D_2O	0.75	21.5	0.150	0.016	0.10
2	" + "	82% "	1.78	30.0	0.250	0.013	0.05
3	" + CH_3CD_2OH	H_2O	2.24	90.1	0.135	0.138	1.02

* In diphenacyl malate.

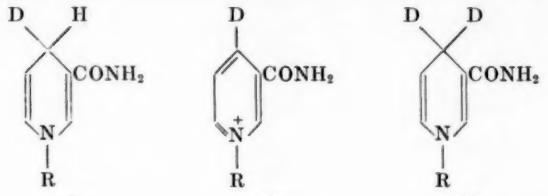
† Calculated for 1 atom of D per molecule of diphenacyl malate.

of D per molecule in Experiment 2, as shown in Table I. These values are far below the minimal figure of 1.0 atom of D per molecule which must be obtained if an enol of OAA were formed in Reaction 1, *c*. The CO_2 fixation must therefore give the keto form of OAA as shown in Path A of Fig. 1. The results also provide further confirmation for the previous conclusion that malic dehydrogenase, also, acts only on the keto form of OAA, since less than 1 atom of non-exchangeable D per molecule of malate can be obtained only if both Reactions 1, *c* and 1, *d* involve the keto acid.

Experiment 3 of Table I was performed to provide further confirmation that the malate was formed in these experiments by the transfer of H from ethanol to oxalacetate by way of DPN. This experiment was carried out in the same way as Experiments 1 and 2, except that CH_3CD_2OH was used instead of unlabeled ethanol, and the medium was H_2O instead of D_2O. The malate formed now contained 1 atom of D per molecule, as expected. The internal consistency of these data, together with those obtained previously in the study of malic dehydrogenase (7), can leave no

doubt of the conclusion that keto-OAA is formed in the irreversible CO_2 fixation reaction.

It might appear at first glance that Experiment 3 also confirms the previous conclusion that both alcohol and malic dehydrogenases have the same steric specificity for DPN (7). This is not correct, however. If DPN is used in catalytic amounts to link two dehydrogenase systems, both of which involve hydrogen transfer to and from DPN, then H must be transferred from the substrate of one dehydrogenase to the substrate of the other by way of DPN, regardless of the relative steric specificities of these reactions. Suppose, for example, that the transfer of D from CH_3CD_2OH to DPN involves the formation of a diastereomer (A). If malic dehydrogenase causes H transfer to and from the opposite side of the pyridine ring, then reoxidation of (A) by OAA will give monodeuterio-oxidized DPN (B). Reduction of (B) by the labeled ethanol must now give a dideuterio-reduced DPN (C), which must obviously transfer D to OAA on reoxidation. If the amount of DPN used is small relative to the amount of OAA reduced, then the amount of OAA reduced by (A) will be small relative to the amounts of OAA reduced by (C). It is therefore necessary to use a stoichiometric amount of (A) for the reduction of OAA in order to determine most easily whether malic dehydrogenase has the same stereospecificity for DPN as does alcohol dehydrogenase (7).



(A) (B) (C)

Fig. 2 shows the mechanism of the non-enzymatic decarboxylation of oxalacetate, according to Steinberger and Westheimer. The enzymatic addition of CO_2 to PEP may be pictured as essentially the reverse of the first step.

Fate of Phosphate—A number of suggestions regarding the possible formation of a phosphoenoloxalacetate have appeared in the literature in various connections (11–14), but none of the hypotheses has received experimental confirmation. Bandurski and Greiner (15) have recently suggested that phosphoenoloxalacetate may be the first product of the irreversible addition of CO_2 to PEP in spinach, and Bartley (16) has suggested that the initial formation of phosphoenoloxalacetate from ATP and OAA may be regarded as a useful working hypothesis to explain the observed

formation of PEP from C_4-dicarboxylic acids. In studies of the ATP-dependent incorporation of $C^{14}O_2$ into the β-carboxyl group of OAA, Utter (17) found that the labeled oxalacetate was not homogeneous but contained a higher activity fraction which decarboxylated more slowly. This derivative was converted to oxalacetate by mild procedures preparatory to chromatography. Later Utter and Kurahashi (18) expressed the view that such a substance might play a rôle as an intermediate in the reversible formation of PEP from OAA and ITP or ATP in the presence of purified liver enzyme. They presented no experimental evidence bearing on the question of whether the oxalacetate derivative contained phosphate.

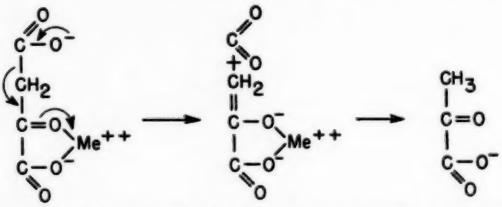
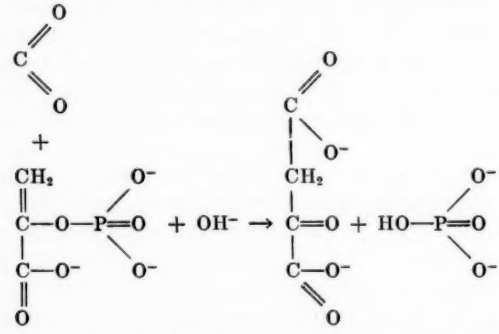


FIG. 2. Mechanism of decarboxylation of oxalacetate. The reaction pictured here shows the metal ion complex which is decarboxylated to the enolate in the metal-catalyzed reaction. The metal ion accelerates the reaction but does not change the basic mechanism.

The fact that oxalacetate appears in the keto form in the enzyme reaction here investigated indicates that in the present case the phosphate-enol P—O bond of PEP must be broken prior to or simultaneously with the CO_2 addition. The phosphate may combine directly with OH^- or H_2O to form free inorganic phosphate as shown in Reaction 2. It is also



(2)

possible, however, that the phosphate might be transferred to one or the other of the two carboxyl groups of OAA, from which it would subsequently

be hydrolyzed, as presented in Fig. 3. Fig. 3 shows how such an oxalacetyl phosphate might also, in a separate reaction, transfer phosphate to an acceptor such as ADP (or IDP) to form ATP (or ITP). Thus, an oxalacetyl phosphate might function as a common intermediate for two carboxylation reactions, one of which is freely reversible and involves a phosphate transfer to IDP or ADP and the other of which is irreversible and involves a liberation of inorganic phosphate. Separate enzymes might catalyze the various steps. Although no direct experimental evidence for the formation of an oxalacetyl phosphate, as pictured in Fig. 3, has yet been found in this laboratory, these negative results are not regarded as conclusive. The data permit a definite conclusion, however, that, if a phosphorylated oxalacetate is formed in the reaction under investigation, then the phosphate must be attached to one of the carboxyl groups. Al-

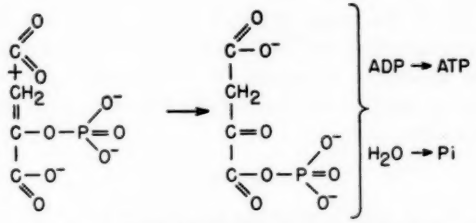


FIG. 3. Hypothetical oxalacetyl phosphate

though this conclusion has been shown to apply specifically only to the irreversible OAA-forming reaction, it seems reasonable to suggest, as a working hypothesis, that the reversible OAA-forming reaction catalyzed by the OAA carboxylase of Utter and Kurahashi (18) also involves the keto form of OAA, and that, under these circumstances, the phosphate of PEP must be split from the molecule or transferred to a carboxyl group when the CO_2 fixation takes place. The properties of an oxalacetyl phosphate should resemble those of the OAA derivative described by Utter (17), since oxalacetyl phosphate would be expected to be readily converted to OAA under mild conditions, and would probably be decarboxylated more slowly than OAA under the experimental conditions employed. Further work is required, however, to confirm or disprove the occurrence of the type of reaction outlined in Fig. 3.

Free Energy Change of Irreversible Carboxylation Reaction—It was of interest to determine whether the observed irreversibility of Reaction 2 was in agreement with the known free energy changes of other reactions. The values of Burton and Krebs (19) were used to calculate the expected

$\Delta F°$ as follows:

$$\text{Enolpyruvate-2-P}^{3-} + \text{ADP}^{3-} + \text{H}^+ \rightarrow \text{pyruvate}^- + \text{ATP}^{4-};\ \Delta F° = -14.5 \text{ kilocalories} \quad (3)$$

$$\text{ATP}^{4-} + \text{H}_2\text{O} \rightarrow \text{ADP}^{3-} + \text{HPO}_4^{2-} + \text{H}^+;\ \Delta F° = +0.9 \text{ kilocalorie} \quad (4)$$

$$\text{Pyruvate}^- + \text{HCO}_3^- \rightarrow \text{oxalacetate}^{2-} + \text{H}_2\text{O};\ \Delta F° = +6.39 \text{ kilocalories} \quad (5)$$

$$\text{Enolpyruvate-2-P}^{3-} + \text{HCO}_3^- \rightarrow \text{oxalacetate}^{2-} + \text{HPO}_4^{2-};\ \Delta F° = -7.21 \text{ kilocalories} \quad (6)$$

$\Delta F°$ ($\Delta G°$ of Burton and Krebs) is the free energy change for unit activity of all components at 25°. $\Delta F°$ for Reaction 6, which is equivalent to Reaction 2, is the sum of the $\Delta F°$ values for Reactions 3, 4, and 5, or -7.21 kilocalories. However, the value for Reaction 3 is not very accurate. On the basis of Utter's measurement of the equilibrium of the reversible, IDP-linked CO_2 fixation reaction, and the assumption that the $\Delta F°$ of hydrolysis of ITP is the same as that for ATP, a value of $\Delta F° = -7.95$ kilocalories has been calculated[2] for Reaction 6. The equilibrium constant calculated from the latter value is $K = 6.6 \times 10^5$. It is clear that this value is sufficiently large to account for the apparent irreversibility of this CO_2 fixation reaction.

SUMMARY

The mechanism of the irreversible enzymatic conversion of PEP and CO_2 into OAA and inorganic phosphate has been studied with the help of deuterium as a tracer. By conducting the reaction in a medium of D_2O, it has been shown that it is the keto form of OAA (and not an enol) which results from the CO_2 fixation. The mechanism is therefore similar to the reverse of the mechanism for the non-enzymatic decarboxylation of β-keto acids. The results also permit the conclusion that, if a phosphorylated derivative of OAA is formed, the phosphate must be attached to a carboxyl group.

The authors are grateful to Professor F. H. Westheimer for his stimulating and helpful discussions.

BIBLIOGRAPHY

1. Pedersen, K. J., *J. Am. Chem. Soc.*, **58,** 240 (1936).
2. Steinberger, R., and Westheimer, F. H., *J. Am. Chem. Soc.*, **73,** 429 (1951).
3. Kornberg, A., Ochoa, S., and Mehler, A. H., *J. Biol. Chem.*, **174,** 159 (1948).
4. Vennesland, B., and Conn, E. E., *Ann. Rev. Plant Physiol.*, **3,** 307 (1952).
5. Tchen, T. T., and Vennesland, B., *J. Biol. Chem.*, **213,** 533 (1955).
6. Vennesland, B., Tchen, T. T., and Loewus, F. A., *J. Am. Chem. Soc.*, **76,** 3358 (1954).

[2] K. Burton, private communication.

XUM

7. Loewus, F. A., Tchen, T. T., and Vennesland, B., *J. Biol. Chem.*, **212,** 787 (1955).
8. Racker, E., *J. Biol. Chem.*, **184,** 313 (1950).
9. Fisher, H. F., Conn, E. E., Vennesland, B., and Westheimer, F. H., *J. Biol. Chem.*, **202,** 687 (1953).
10. Stafford, H. A., and Vennesland, B., *Arch. Biochem. and Biophys.*, **44,** 404 (1953).
11. von Euler, H., and Malmberg, M., *Ark. Kemi, Mineral. o. Geol.*, **12 B,** 6 (1936).
12. Lipmann, F., *Advances in Enzymol.*, **1,** 99 (1941).
13. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140,** 171 (1941).
14. Meyerhof, O., A symposium on respiratory enzymes, Madison, 3 (1942).
15. Bandurski, R. S., and Greiner, C. M., *J. Biol. Chem.*, **204,** 781 (1953).
16. Bartley, W., *Biochem. J.*, **56,** 387 (1954).
17. Utter, M. F., *J. Biol. Chem.*, **188,** 847 (1951).
18. Utter, M. F., and Kurahashi, K., *J. Biol. Chem.*, **207,** 821 (1954).
19. Burton, K., and Krebs, H. A., *Biochem. J.*, **54,** 94 (1953).

THE METABOLIC CONTROL OF HISTIDINE ASSIMILATION AND DISSIMILATION IN AEROBACTER AEROGENES*

By BORIS MAGASANIK

(From the Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts)

(Received for publication, August 13, 1954)

In the course of our investigation of the ability of auxotrophic mutants of *Aerobacter aerogenes* to adapt to the degradation of *myo*-inositol, the unexpected observation was made that a histidineless mutant, strain 50, required approximately 25 times as much L-histidine to achieve full growth on *myo*-inositol as was necessary for full growth on glucose (2). This finding prompted the study of the effect of the composition of the growth medium on the histidine requirement of the organism. The results, presented in the present paper, reveal that the amount of histidine which has to be supplied to permit the growth of the mutant depends on the balance between assimilation and dissimilation of histidine during growth. Some of the mechanisms which control this balance were elucidated and correlated with the effect of the chemical composition of the environment on the enzymatic composition of the organism.

Materials and Methods

Bacteria—The origin and method of cultivation of *A. aerogenes*, strain 1033, and of mutants prepared from it have been described previously (2). Histidineless and tryptophanless mutants of *Escherichia coli*, kindly supplied by the late Dr. J. H. Mueller, were used in some experiments.

Chemicals—*Acetyl-L-histidine*, *L-glycyl-L-histidine*, and *carnosine* (β-alanyl-L-histidine) were gifts of the late Dr. J. H. Mueller. *L-Histidine* and *DL-tryptophan* were Merck preparations. The purity of these products was verified by paper chromatography. *myo-Inositol* was obtained from the Corn Products Refining Company. The various sugars used were of chemically pure grade.

Analytical Methods—The *growth response* of the organisms and their *growth rate* under different conditions were determined by the methods described previously (2). *Oxygen uptake* was measured by conventional Warburg methods (3). When limited amounts of resting cells were available, Warburg vessels of 7 ml. capacity were used. *Amino acids* and *pep-*

* This work has been supported in part by a grant from the United States Public Health Service and by funds received from the Eugene Higgins Trust. A preliminary report of this work has been published (1).

XUM

tides were separated by paper chromatography in a solvent system consisting of 95 parts of *n*-butanol and 5 parts of formic acid, one-half saturated with water, and located on the dried paper by spraying with ninhydrin dissolved in *n*-butanol and heating. *Histidine* and other *imidazole* derivatives could be identified on the chromatograms by spraying with an alcoholic solution of *p*-anisidine and isoamyl nitrite, followed by treatment with ammonia vapor or alcoholic potassium hydroxide (4). *Histidine* and its *acyl derivatives* were quantitatively determined in culture fluids by the method of Jorpes (5). *Glucose* was determined as reducing sugar (6).

Results

Histidine Requirement of Strain 50 on Different Carbon Sources—The growth response of strain 50 to L-histidine was determined in media containing a variety of sugars, alcohols, and organic acids as sole sources of carbon. The results, summarized in Table I, show that poor utilization of histidine in a medium containing *myo*-inositol is by no means an isolated phenomenon. The compounds tested as carbon sources can be divided rather sharply into two groups: those similar to glucose, on which almost full growth can be attained with a supplement of 20 γ of histidine, and those similar to *myo*-inositol, constituting the larger group, on which more than 100 γ of histidine are required for full growth. However, considerable variation occurs within the groups. No other compound was found to equal glucose in permitting full growth with less than 20 γ of histidine. The observed effects cannot easily be correlated with any obvious chemical features of the compounds tested. A relationship does, however, seem to exist between the response to histidine and the rate of growth of the organism on a particular compound;[1] those which support rapid growth of the organism permit the most efficient utilization of histidine.

The requirement for L-histidine was not noticeably influenced by changing the components of the medium other than the source of carbon. The addition of glycine, tyrosine, arginine, glutamic acid, adenine, or uracil singly or the addition of a mixture containing glutamic acid, aspartic acid, lysine, proline, pantothenic acid, *p*-aminobenzoic acid, thiamine, pyridoxine, biotin, riboflavin, and folic acid did not change the response of the mutant to histidine on either glucose or inositol; similarly, no difference was noted when an acid hydrolysate of casein (Bacto-casamino acids, Difco) in a concentration sufficient to provide 20 or 100 γ of L-histidine per ml. was used in place of L-histidine alone. Furthermore the response to histidine on glucose and on inositol was not influenced by changes of pH between 5 and 8, under either aerobic or anaerobic conditions.

[1] These experiments were suggested by Dr. B. D. Davis and carried out by Mr. Frederick C. Neidhardt.

Replacement of Histidine by Acylhistidines—Histidine could be replaced as a growth factor by acetyl-L-histidine, L-glycyl-L-histidine, or carnosine (β-alanyl-L-histidine). The growth response of the mutant to these compounds in a medium containing glucose or *myo*-inositol as the source of carbon is illustrated in Fig. 1. It can be seen that on glucose the organism responds equally well to histidine, acetylhistidine, and glycylhistidine and somewhat less well to carnosine. On *myo*-inositol, on the other hand, the organism responds very much better to the acyl derivatives of histidine than to free histidine. The growth in an inositol medium supplemented

TABLE I

Utilization of L-Histidine by A. aerogenes, Strain 50, for Growth on Different Sources of Carbon

The organism was grown in minimal medium containing the carbon source in a final concentration of 0.2 per cent and L-histidine in a concentration of 20 γ, 100 γ, and 500 γ per ml., respectively, at pH 6.0 with aeration for 18 hours at 37°. Growth was measured spectrophotometrically. The results are expressed in per cent of the growth unrestricted by insufficient nutrilite, that is of the growth with a supplement of 500 γ of L-histidine.

Carbon source	Per cent of unrestricted growth*		Generation time,† min.
	20 γ L-histidine	100 γ L-histidine	
Glucose	109	108	51
Gluconic acid	88	103	50
Galactose	67	94	
Mannitol	66	115	50
Arabinose	66	123	
Rhamnose	16	77	
Sorbitol	11	66	
Dihydroxyacetone	17	64	
Succinic acid	13	61	
Fructose	20	60	
Fucose	7	59	
myo-Inositol	12	58	64
Glycerol	8	48	
Ribose	4	48	
Mannose	9	46	77
Pyruvic acid	13	43	95
Xylose	6	32	
Lactic acid	9	27	

* Corrected for the growth obtained in the absence of the carbon source.

† In these experiments, carried out by Mr. F. C. Neidhardt, the parent strain 1033 was used.

with acetylhistidine is almost as good as that in a corresponding glucose medium. Glycylhistidine is utilized somewhat less efficiently on inositol than on glucose. Of interest is the inverse relationship which was found to exist between the ability of histidine and acylhistidines to support growth on inositol and their ability to serve as sole sources of carbon for the growth of the organism; good growth was obtained on histidine alone, fair growth on glycylhistidine, and no growth at all on acetylhistidine or carnosine.

Hydrolysis of Acylhistidines (7)—Resting cell suspensions of strain 50, grown on glucose in a medium supplemented with histidine, were incubated

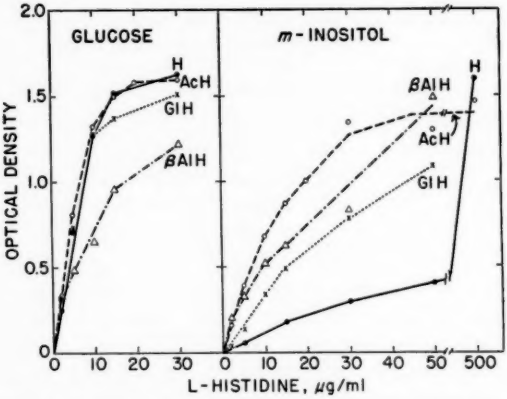


FIG. 1. Growth response of *A. aerogenes*, strain 50, to L-histidine and acylhistidines in a medium containing glucose or *myo*-inositol as the major source of carbon. *H*, L-histidine; *AcH*, acetyl-L-histidine; *GlH*, L-glycyl-L-histidine; *βAlH*, carnosine.

in 1 ml. of 0.067 M phosphate buffer of pH 6, containing 10 μmoles of glycylhistidine or acetylhistidine, at 37° for 2 hours, in an atmosphere of air or of nitrogen. The bacteria were removed by centrifugation and the supernatant fluids subjected to paper chromatography in a butanol-formic acid solvent. The production of glycine (R_f 0.27) from glycylhistidine (R_f 0.14) could be demonstrated under aerobic and anaerobic conditions. Similarly, the production of histidine (R_f 0.12) could be demonstrated after the anaerobic incubation of the cell suspension with acetylhistidine (R_f 0.48). In the presence of air no histidine accumulated, but colorimetric assay for the imidazole group (5) revealed the disappearance of about one-fifth of the added acetylhistidine. Similar results were obtained when cells dried *in vacuo* over P_2O_5 were used in place of resting cells.

Disappearance of Histidine during Growth—The observation that on

inositol the histidine requirement of the mutant can be reduced more than 10-fold by supplying the nutrilite in the form of acetylhistidine indicates that this smaller amount is sufficient to furnish the organism with the histidine necessary for the synthesis of bacterial protein. The need of the

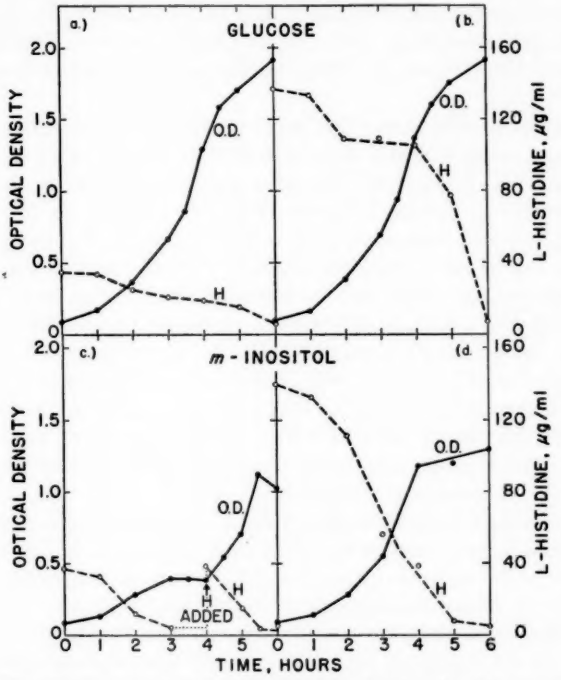


FIG. 2. The disappearance of histidine during the growth of *A. aerogenes*, strain 50, on glucose or *myo*-inositol. *O. D.*, optical density of the culture; *H*, concentration of L-histidine. The medium, containing glucose (*a*, *b*) or *myo*-inositol (*c*, *d*) as the major carbon source and a supplement of 40 γ (*a*, *c*) or 150 γ (*b*, *d*) of L-histidine per ml., was inoculated with a culture grown 18 hours on inositol supplemented with 20 γ per ml. of acetyl-L-histidine.

organism growing on inositol and free histidine for amounts of histidine in excess of that required for protein synthesis must be due either to the inability of the exogenous histidine to penetrate the cell in the absence of a steep concentration gradient (7) or, alternatively, to excessive destruction of free histidine. The experiments illustrated in Fig. 2 indicate that histidine disappears from the medium very rapidly when inositol is the source of carbon. The cessation of growth is clearly seen to be a consequence of the

exhaustion of the histidine. Comparison of Fig. 2, *c* and *d* reveals the absence of a quantitative correlation between the extent of growth on inositol and the amount of histidine used; thus, growth on a total of 80 γ of histidine supplied in two portions nearly equals the growth on a single portion of 150 γ of histidine. During growth on glucose, histidine disappearance is slow, irrespective of the amount of histidine present in the medium. However, after exhaustion of the glucose, indicated by a decrease in the rate of growth (Fig. 2, *b*), histidine disappearance becomes extremely rapid.

Degradation of Histidine—The rapid destruction of histidine by cells growing on inositol indicates the presence of an enzyme system catalyzing the degradation of histidine; the persistence of histidine in the medium during growth on glucose indicates either the absence of the histidine-degrading enzymes from such cells or the inhibition of their activity by glucose or one of its degradation products. Preliminary experiments revealed that resting cells grown on histidine alone, or on inositol supplemented with 500 γ per ml. of histidine, could degrade histidine, with the uptake of oxygen and the production of CO_2. Cells grown on inositol supplemented with acetylhistidine or on glucose supplemented with histidine were only weakly active against histidine. Similar results were obtained when the parent strain 1033 was used; cells grown on histidine alone oxidized histidine, while cells grown on glucose alone or inositol alone were inactive. Histidine-grown washed cells, dried *in vacuo* over P_2O_5, could oxidize histidine. Similar preparations of glucose-grown cells were inactive and could not be activated by the addition of extracts of "boiled" histidine-grown cells. The enzymes responsible for the degradation of histidine are thus seen to be adaptive enzymes whose synthesis is induced by histidine.

The pathway of histidine degradation was investigated. The results presented in the following paper (8) indicate that histidine is converted without the uptake of oxygen to formamide, ammonia, and glutamic acid; the last compound is oxidized. The steps leading to glutamic acid are sufficiently rapid to permit the measurement of oxygen uptake to be used as a measure of histidase activity (8).

Biosynthesis of Histidase—The effect of inositol and of glucose on the rate of synthesis of histidase was studied by inoculating cells grown on histidine alone, or on inositol with histidine or acetylhistidine, into media containing histidine, glucose, and inositol in different combinations. The results of some of these experiments are illustrated in detail in Fig. 3, where it can be seen that histidase production proceeds linearly with growth on inositol, but is almost completely absent on glucose. When the glucose concentration of the medium has been reduced to about one-tenth (which occurred after 4 hours in this experiment), the growth rate declines sharply and the rapid synthesis of histidase begins. The disappearance of histidine

during growth on inositol is well correlated with the presence of histidase in these cells. On glucose the histidine level drops sharply during the first 2 hours of growth, although the cells are almost devoid of histidase (see also Fig. 2, *b*). This period is followed by a 4-fold increase in cell mass during the next 2 hours, with almost no uptake of histidine. It would therefore appear that a portion of the histidine removed from the medium during the first 2 hour period is stored in the cells in a form available for subsequent protein synthesis. The rapid disappearance of histidine from

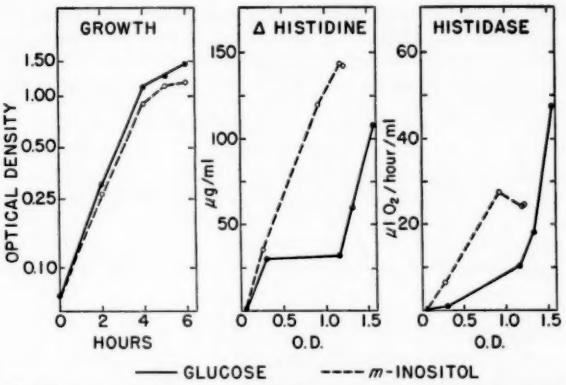


FIG. 3. Histidase production and histidine disappearance during the growth of *A. aerogenes*, strain 50, on glucose or *myo*-inositol. The medium contained 150 γ of histidine per ml. The experimental details are described in Table II, Experiments 1 and 3.

the medium after exhaustion of the glucose is well correlated with the increased histidase content of these cells.

The experiments summarized in Table II show that glucose inhibits the rate of histidase production, measured as the ratio of the amount of enzyme formed to the total amount of new protoplasm synthesized (last column), even when the histidine concentration of the medium is increased to equal that of the glucose (Experiments 2 and 8). Glucose seems to inhibit the histidase production of cells previously adapted to histidine (Experiments 6 and 8), as well as of cells unadapted to histidine (Experiments 1 and 2).

It can further be seen that inositol is not completely without effect on histidase production. Cells growing on inositol and histidine (Experiments 3, 4, 7, and 9) produce only about one-half as much histidase as cells growing on histidine alone (Experiments 5 and 10). The inhibitory action of inositol, like that of glucose, does not seem to be greatly influenced by the histidine content of the medium or by the previous history of the culture.

It is of interest that histidine, in its turn, does not affect at all the pro-

TABLE II

Synthesis of Histidase and of Inositol Dehydrogenase by A. aerogenes, Strain 50

The cells were grown in minimal medium containing the compound serving as carbon source (2000 γ per ml.) and the histidine supplement indicated in the second and third columns. A volume of an 18 hour culture grown as indicated, sufficient to give an optical density of about 0.1 at 590 mμ, served as inoculum. The enzyme activities of washed cells from the inoculum and from the growth medium at the end of logarithmic growth (usually 4 hours) were determined by measuring their oxygen uptake in air, in a total volume of 1.2 or 3.0 ml. of 0.067 M phosphate buffer of pH 6, at 37° for a period of 20 minutes. The vessels, of 7 or 15 ml. capacity, contained substrate, L-histidine, or *myo*-inositol, in a concentration of 1.66×10^{-3} M, and sufficient cells to give an optical density of about 2.0 at 590 mμ. The enzyme activities are expressed in microliters of O_2 (corrected for endogenous respiration) taken up in 1 hour.

Experiment No.	Carbon source	Histidine	Generation time	Histidase			Inositol dehydrogenase		
				Initial*	Final*	Differential rate of synthesis†	Initial*	Final*	Differential rate of synthesis†
Inoculum grown on 2000 γ of *myo*-inositol and 20 γ of acetyl-L-histidine per ml.									
		γ per ml.	min.						
1	Glucose	150	58	4.0	8.9	9			
2	"	2000	60	3.5	8.6	9	63.5	3.2	0
3	Inositol	150	64	4.0	30.7	33			
4	"	2000	71	3.5	43.1	47	63.5	72.6	74
5	None	2000	84	3.5	75.0	86	63.5	58.8	58
Inoculum grown on 2000 γ of *myo*-inositol and 500 γ of L-histidine per ml.									
6	Glucose	150	60	31.0	6.6	3			
7	Inositol	150	67	31.0	36.9	38			
Inoculum grown on 2000 γ of L-histidine per ml.									
8	Glucose	2000	63	84.5	20.0	15	2	2	0
9	Inositol	150	69	84.5	33.3	28	2	68.7	75
10	None	2000	73	84.5	78.2	100	2	2	0

* Per unit of cells. A unit of cells is defined as the number of cells contained in 1.0 ml. of a suspension of an optical density of 1.0 at 590 mμ determined in a square cuvette (light path 13 mm.) in a Coleman universal spectrophotometer, model 14 (9).

† The increase in enzyme activity per ml. of culture divided by the increase in optical density of the culture (10).

duction of inositol dehydrogenase, which like histidase is an adaptive enzyme (11, 12). Thus cells grown on inositol and acetylhistidine, and consequently fully adapted to inositol but not to histidine, maintained their level of inositol dehydrogenase activity during growth on a mixture of inositol and histidine (Experiment 4). In fact, these inositol-adapted cells continued to produce inositol dehydrogenase during growth on histidine in the absence of inositol (Experiment 5). However, no inositol dehydrogenase was produced when the cells were cultured in the presence of glucose (Experiment 2). Similarly, cells adapted to histidine but not to inositol, growing on a mixture of histidine and inositol, synthesized inositol dehydro-

TABLE III

Utilization of L-*Histidine and of* L-*Tryptophan by Auxotrophic Mutants of E. coli for Growth on Glucose and on Succinic Acid*

The experimental procedure is similar to that described in Table I. The growth response is expressed in per cent of growth unrestricted by insufficient nutrilite; that is, in this case, of the growth obtained with a supplement of 100 γ of nutrilite.

Mutant	Carbon source	Supplement	Per cent of unrestricted growth, 20 γ nutrilite	Indole* formed
Histidineless	Glucose	L-Histidine	100	
"	Succinic acid	"	147†	
"	Glucose	Acetyl-L-histidine	99	
"	Succinic acid	"	97	
Tryptophanless	Glucose	Tryptophan	86	+
"	Succinic acid	"	10	+++

* Estimated by the method of Kovács (14).

† Histidine in high concentrations is inhibitory to the organism growing on succinic acid.

genase so rapidly that after 2 hours their content of this enzyme equaled that of inositol-grown cells (compare Experiments 4 and 9).

Growth on Different Carbon Sources of Tryptophanless and Histidineless E. coli Mutants—These experiments were carried out in order to demonstrate in another system the correlation between the dissimilation of a nutrilite by adaptive enzymes during growth on carbon sources other than glucose and an increased requirement for that nutrilite. The mutants of *A. aerogenes* examined previously (2), with the exception of the histidineless mutant discussed here, all grew equally well on glucose and inositol. *A. aerogenes* does not possess the enzyme *tryptophanase* which is responsible for the dissimilation of tryptophan, with the production of indole in *E. coli* (13). Consequently, the efficient utilization of tryptophan by a tryptophanless mutant of *A. aerogenes* growing on inositol (2) is not surprising.

The experiments in which histidineless and tryptophanless mutants of *E. coli* were used show a different pattern of behavior. In these experiments, summarized in Table III, glucose and succinic acid were used as the sources of carbon, because *E. coli* cannot grow on *myo*-inositol. It can be seen that histidineless *E. coli* responds equally well to histidine on glucose and on succinic acid. In contrast to *A. aerogenes*, *E. coli* is incapable of growing on histidine as the sole source of carbon. This organism does not seem to be endowed with the ability to form the enzymes necessary for the degradation of histidine, and consequently the histidine supplied can be used efficiently for protein synthesis. The tryptophanless *E. coli* mutant, however, requires more tryptophan for growth on succinic acid than for growth on glucose. The accumulation of indole in the succinic acid medium indicates that the increased need for tryptophan arises from an increased degradation of tryptophan by tryptophanase.

DISCUSSION

The experimental results presented in this paper provide a clear explanation for the original observation (2) that the histidineless mutant of *A. aerogenes* requires more histidine for growth on inositol than for growth on glucose. The dissimilation of histidine by adaptive enzymes during growth on inositol reduces the quantity of histidine available for protein synthesis and consequently increases the demand of the organism for histidine; this wasteful dissimilation of added histidine does not occur during growth on glucose (Fig. 2). The study of the kinetics of the histidine-induced biosynthesis of histidase in a medium containing glucose, inositol, or histidine alone shows clearly that glucose exerts its effect by inhibiting the rate of synthesis of histidase relative to the rate of synthesis of the other components of the bacterial protoplasm (Fig. 3, Table II).

The inhibitory effect of carbohydrates on the production of certain adaptive enzymes has long been known. The outstanding examples are the phenomenon of "diauxie," that is of the stepwise growth of bacteria on a mixture of two carbohydrates (15), and the suppression of the formation of bacterial amino acid deaminases during growth on glucose (16). The observations reported here demonstrate that this effect can be of importance for the economy of the growing cell. It enables the organism to use histidine almost exclusively for the purpose for which it is best suited, the synthesis of protein, as long as glucose is available as a source of energy and undifferentiated building blocks; on the other hand in a medium rich in histidine but not containing glucose, such as a protein hydrolysate, the ability to synthesize histidase enables the organism to use histidine as its source of energy, carbon, and nitrogen.

It is puzzling that compounds capable of serving as sole sources of carbon

for the growth of *A. aerogenes* differ greatly in their ability to suppress the biosynthesis of histidase. Only a small number of the compounds tested are comparable to glucose in potency; the majority resemble inositol in being only weakly inhibitory (Table I). The compounds of the first group have no apparent structural regularity in common which would explain their activity. It seems, however, that the ability to suppress the biosynthesis of adaptive enzymes is a feature peculiar to these compounds and not restricted to this particular enzyme system and organism. Thus, in *E. coli* glucose, gluconic acid, mannitol, arabinose, and fructose inhibit the formation of indole from tryptophan by suppressing the synthesis of tryptophanase; rhamnose, sorbitol, ribose, xylose, and galactose, on the other hand, have no inhibitory effect on indole production (17). Inspection of Table I reveals that with the exception of only fructose and galactose these compounds act in the same fashion on the biosynthesis of histidase. The similarity in the metabolic importance of the control of histidase and tryptophanase production is emphasized by the demonstration that the composition of the growth medium affects equally the tryptophan requirement of a tryptophanless mutant of *E. coli* (Table III) and the histidine requirement of the histidineless mutant of *A. aerogenes* (Table I).

The mechanism by which glucose suppresses the biosynthesis of histidase is not known. Cohn and Monod have recently suggested that the inhibitory action of glucose on the synthesis of adaptive enzymes results from its ability to prevent the penetration of the cell by the inducer (18). This explanation cannot be applied to the present case. The observation that during the early stage of growth histidine disappears from the medium as rapidly in the presence of glucose as in its absence (Figs. 2 and 3) indicates that here glucose does not render the cell impermeable to histidine; yet even during this period glucose inhibits the production of histidase (Fig. 3). The inhibition of adaptive enzyme synthesis has also been ascribed to the competition among different enzyme-forming systems for the building blocks necessary for the synthesis of protein (19). In that case, increasing the supply of such building blocks should lift the restriction of protein synthesis; however, glucose inhibits histidase production even in a medium enriched with casein hydrolysate.

The observation that the compounds supporting the most rapid growth are also the most potent inhibitors of histidase production seems significant. At present the nature of the particular metabolic reaction which limits the rate of growth is not known; its elucidation may offer an explanation of the mechanism responsible for the control of adaptive enzyme synthesis.

Snell and his collaborators have described and analyzed a variety of conditions in which peptides surpass free amino acids in growth-promoting ability (20–22, 7). Applying similar reasoning to the present case reveals

that the growth-promoting action of the acylhistidines, which for cells growing on inositol exceeds by far that of free histidine (Fig. 1), cannot be due to the direct incorporation of these compounds into protein, as the acid with which histidine is combined in the two most active compounds (acetic acid or β-alanine) is not a component of protein. It is possible that histidine is directly transferred from these compounds to other peptidic combinations. In this case, owing to the absence of free histidine, no biosynthesis of histidase would occur, and, even if it were formed, the enzyme would not be able to destroy histidine bound in a peptidic linkage. However, so far no reaction mechanism is known by which histidine occupying the carboxyl end of a peptide, as in an acylhistidine, could be directly transferred to other peptides. The ability of resting and dried cell preparations of this organism to hydrolyze acetylhistidine and glycylhistidine suggests that free histidine is an intermediate in the synthesis of protein from acylhistidines. The release of free histidine from the acyl linkage may occur so gradually that at no time a level of histidine sufficiently high to induce the biosynthesis of histidase is attained in the cell. Furthermore the ratio of histidine assimilation and dissimilation seems to be higher at low levels of histidine; thus histidine supplied to the organism in several small portions is more effectively utilized than histidine supplied in one large portion (Fig. 2, *c* and *d*). A higher affinity for histidine of the protein-synthesizing enzymes than of the degradative enzymes would explain this effect.

Poor utilization for growth because of excessive dissimilation is the cause of the very high tyrosine requirement of *Streptococcus faecalis* growing in a medium rich in vitamin B_6 (21). In that case the composition of the growth medium controls the degradation of the nutrilite by determining the level of the coenzyme of the degradative enzyme. The results presented in this paper reveal another mechanism for the control of the balance of assimilation and dissimilation: the chemical composition of the growth medium determines the rate of degradation of the nutrilite by controlling the rate of synthesis of the responsible apoenzyme.

SUMMARY

The amount of L-histidine required to support the growth of a histidineless mutant of *Aerobacter aerogenes* depends on the nature of the major carbon source of the medium. A few compounds, of which glucose is the most prominent, permit full growth with a supplement of 20 γ per ml. of L-histidine. The majority of the compounds which can serve as sole sources of carbon for *A. aerogenes* requires a supplement of about 500 γ per ml. of the nutrilite. The cause of this phenomenon was investigated with glucose and *myo*-inositol as carbon sources representative of the two classes

of compounds. It was found that during growth on inositol the added histidine was rapidly lost through degradation by adaptive enzymes whose synthesis it had induced. Glucose was discovered to be a powerful inhibitor of the synthesis of these enzymes. Acyl derivatives of L-histidine could supply histidine for protein synthesis, but did not induce the production of histidine-degrading enzymes; consequently the growth-promoting activity of acylhistidines on inositol exceeded by far that of free histidine. These results show that the ratio of histidine assimilation and dissimilation depends on the concentration of histidine-degrading enzymes in the cell, which in turn is controlled by the composition of the growth medium.

I am grateful to Miss Doris Karibian for excellent assistance.

BIBLIOGRAPHY

1. Magasanik, B., *Federation Proc.*, **12**, 241 (1953).
2. Ushiba, D., and Magasanik, B., *Proc. Soc. Exp. Biol. and Med.*, **80**, 626 (1952).
3. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1946).
4. Sanger, F., and Tuppy, H., *Biochem. J.*, **49**, 463 (1951).
5. Jorpes, E., *Biochem. J.*, **26**, 1507 (1932).
6. Park, J. T., and Johnson, M. J., *J. Biol. Chem.*, **181**, 149 (1949).
7. Peters, V. J., Prescott, J. M., and Snell, E. E., *J. Biol. Chem.*, **202**, 521 (1953).
8. Magasanik, B., and Bowser, H. R., *J. Biol. Chem.*, **213**, 571 (1955).
9. Magasanik, B., Brooke, M. S., and Karibian, D., *J. Bact.*, **66**, 611 (1953).
10. Monod, J., Pappenheimer, A. M., Jr., and Cohen-Bazire, G., *Biochim. et biophys. acta*, **9**, 648 (1952).
11. Magasanik, B., *J. Biol. Chem.*, **205**, 1007 (1953).
12. Goldstone, J. M., and Magasanik, B., *Federation Proc.*, **13**, 218 (1954).
13. Happold, F. C., *Advances in Enzymol.*, **10**, 51 (1950).
14. Kovács, N., *Z. Immunitätsforsch. u. exp. Therap.*, **55**, 311 (1928).
15. Monod, J., Recherches sur la croissance des cultures bactériennes, Paris (1942).
16. Epps, H. M. R., and Gale, E. F., *Biochem. J.*, **36**, 619 (1942).
17. Evans, W. C., Handley, W. R. C., and Happold, F. C., *Biochem. J.*, **36**, 311 (1942).
18. Cohn, M., and Monod, J., in Gale, E. F., and Davies, R., Adaptation in microorganisms, Cambridge (1953).
19. Spiegelman, S., and Dunn, R., *J. Gen. Physiol.*, **31**, 153 (1947).
20. Kihara, H., and Snell, E. E., *J. Biol. Chem.*, **197**, 791 (1952).
21. Kihara, H., Klatt, O. A., and Snell, E. E., *J. Biol. Chem.*, **197**, 801 (1952).
22. Prescott, J. M., Peters, V. J., and Snell, E. E., *J. Biol. Chem.*, **202**, 533 (1953).

THE DEGRADATION OF HISTIDINE BY AEROBACTER AEROGENES*

By BORIS MAGASANIK and HELEN R. BOWSER

(From the Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts)

(Received for publication, August 13, 1954)

In the course of the studies presented in the preceding paper it was observed that *Aerobacter aerogenes* could grow on histidine as the sole source of carbon and nitrogen (1). The nature of the microbial enzymes responsible for the complete degradation of histidine was not known and appeared therefore to be an appropriate subject for investigation. After initiation of these studies, the results of the investigations of Tabor and his collaborators on the degradation of histidine by *Pseudomonas fluorescens* were published (2, 3). It became apparent that the histidine metabolism of *A. aerogenes* and of *P. fluorescens* followed the same general pattern (also observed in mammalian liver (4)), yet with significant differences. The present paper describes the dissimilation of histidine in *A. aerogenes* and compares the pathways of histidine degradation of the two microorganisms.

EXPERIMENTAL

Materials

Chemicals—L-*Histidine*, L-*histidine monohydrochloride*, and L-*glutamic acid* were products of the Nutritional Biochemicals Corporation, Cleveland, Ohio. In some of the early experiments, urocanic acid prepared enzymatically (5) and kindly given to us by Dr. Herbert Tabor was used. The urocanic acid in most experiments was synthesized by the method of Edlbacher and von Bidder (6); it had a melting point of 224–226°, identical with that reported in the literature (5), and an absorption peak (ϵ = 16,660) at 272.5 mμ in 0.067 M phosphate buffer of pH 6.3. *N-Formyl-*L-*glutamic acid* was a kind gift of Dr. Herbert Tabor. A synthetic sample of L-α-*formamidinoglutaric acid* was generously provided by Dr. Heinrich Waelsch.

Bacteria—The cultivation of *A. aerogenes*, strain 50 (histidineless), has been described previously (7). The major carbon source of the medium was either glucose supplemented with 15 γ of histidine per ml. or L-histi-

* This work has been supported in part by a grant from the United States Public Health Service and by funds received from the Eugene Higgins Trust. A preliminary report was presented before the Society of American Bacteriologists, San Francisco, California, August, 1953.

XUM

dine hydrochloride in a concentration of 0.2 per cent. *P. fluorescens*, strain 6, a culture of which was kindly supplied by Dr. Herbert Tabor, was grown in a medium containing 0.2 per cent L-histidine hydrochloride supplemented with 0.1 per cent Bacto-yeast extract (Difco) (2).

Methods

Preparation of Resting Cells—Resting cell suspensions of *A. aerogenes*, strain 50, were prepared as described previously (7).

Preparation of Dried Cells—The cells grown as described above were harvested by centrifugation and washed once in one-tenth of the original volume of cold 0.067 M phosphate buffer of pH 6.0. The cell paste was spread in a thin layer over the inside of the centrifuge tubes, and these were placed over P_2O_5 in a vacuum desiccator which was evacuated by an oil pump at room temperature for a period of 3 hours. The unopened desiccator was kept at 4° for 18 hours before the dried cell powder was removed and used immediately or stored at −20°.

Preparation of Cell-Free Extracts—4 liter cultures of *A. aerogenes* or *P. fluorescens* were grown in the proper medium with aeration for 18 hours. The bacteria were harvested by centrifugation at 4° and washed with 500 ml. of 0.01 M phosphate buffer of pH 6.3. The yield was approximately 1.5 gm. of wet bacteria per liter of culture. 6 gm. of cells were ground with 12 gm. of aluminum oxide for 10 minutes at 4°. The mixture was extracted with 20 ml. of 0.01 M phosphate buffer of pH 6.3, and the aluminum oxide and cellular débris were removed by centrifugation at 18,000 × *g* at 0° for 40 minutes.

The extracts prepared from *A. aerogenes* grown on histidine had urocanase activity (5); 0.1 ml. of extract (containing about 0.6 mg. of protein) catalyzed the destruction of 7 μmoles of urocanic acid in 30 minutes in a test system containing 10 μmoles of urocanic acid in a total volume of 1.0 ml. of 0.067 M phosphate buffer of pH 7.0 at 37°. The activity of the extract decreased very little upon storage at −20° for 4 months. The extracts prepared from *P. fluorescens* had similar urocanase activity, but were considerably less stable; their activity was almost completely lost after 5 days storage at 4° or at −20°.

Analytical Procedures—Two solvent systems were used for the separation and identification of histidine and some of its degradation products by ascending *paper chromatography*. *Solvent I* consisted of *sec*-butanol, formic acid, and water, 19:2:6, and was freshly prepared before use. *Solvent II* consisted of *sec*-butanol and concentrated ammonium hydroxide, 9:1, saturated with water.

L-*Histidine* was identified by paper chromatography (Solvent I, R_F 0.14; Solvent II, R_F 0.25) (8) and estimated colorimetrically by the method of Jorpes (9).

Urocanic acid could be detected on paper chromatograms illuminated by ultraviolet light (Mineralight) as a dark spot (Solvent I, R_F 0.69; Solvent II, R_F 0.32) and estimated spectrophotometrically by its ultraviolet extinction (5). This compound reacts in the colorimetric procedure used for histidine determination (9); consequently the values obtained for histidine were corrected for the urocanic acid present in the mixtures.

Glutamic acid was detected on paper chromatograms by spraying with a solution of 1 per cent ninhydrin in ethanol and heating (Solvent I, R_F 0.40; Solvent II, R_F 0.09). For quantitative estimation, glutamic acid was separated from other ninhydrin-reactive substances by paper chromatography in Solvent I and its position located by light spraying with ninhydrin solution and heating; the appropriate areas of the chromatogram as well as blank areas were cut out, contaminating ammonia was removed by treatment with potassium hydroxide as described by Fowden (10), and the glutamic acid eluted and estimated with the ninhydrin reagent of Moore and Stein (11), as described by Awapara and Seale (12).

L-α-*Formamidinoglutaric acid* could be identified by chromatography in Solvent I and treatment with ninhydrin (R_F 0.48); the characteristic purple spot did not appear immediately after heating, but only after a delay of about 1 week.

Glutamine reacted with ninhydrin without delay and was characterized by an R_F value of 0.15 in Solvent I.

Ammonia was determined according to the microdiffusion method of Conway (13). Two procedures were used to estimate *free ammonia* and *alkali-labile (amide) ammonia*. In the first of these, which was used in the experiments with resting cells, the free ammonia was released by treatment with saturated potassium carbonate; both free and alkali-labile ammonia was released by treatment with 20 per cent KOH (final concentration). The ammonia (0.2 to 5 μmole) was collected in boric acid and titrated with 0.1 N acid from a Gilmont ultramicro burette (Emil Greiner Company). It was found that some samples of commercial potassium carbonate caused a partial hydrolysis of amides. Consequently, in later experiments with cell-free extracts the total (free plus amide) ammonia was determined as before; another aliquot of the sample was treated with Permutit to effect the quantitative removal of free ammonia, and the alkali-labile ammonia of the supernatant fluid was determined directly. The methods were tested with ammonia, formamide, and glutamine and were found to be precise.

Formic acid was distilled from the acidified reaction mixture frozen in the still described by Grant (14) and estimated colorimetrically in the distillate (15). *Alkali-labile formic acid*, as in formamide or L-α-formamidinoglutaric acid, was liberated by treatment with alkali at pH 14 for 1 hour at 37°, the mixture acidified, and the formic acid determined as before.

Oxygen uptake and *CO_2 evolution* were determined by conventional manometric procedures (16).

Results

Degradation of L-Histidine and of L-Glutamic Acid by Resting Cell Suspensions of A. aerogenes—The ability of the cells to oxidize histidine or glutamic acid depends on the medium in which they have been grown. Histidine-grown cells oxidize both amino acids at a rapid rate, while glucose-grown cells are devoid of activity. Cells grown on glutamic acid can oxidize glutamic acid, but not histidine. During the early stages of histidine degradation small amounts of urocanic acid and of glutamic acid are found in the reaction mixture; these disappear as the oxidation progresses (Table I, Experiments 1 and 2). The oxidations were permitted to continue until the rate of oxygen uptake had returned to the endogenous levels (Table I, Experiments 3 and 4); the total amounts of oxygen taken up and of CO_2 produced were the same when histidine and glutamic acid were substrates and correspond to about one-half of the theoretical values for the complete oxidation of glutamic acid to CO_2, H_2O, and NH_3. Apparently a fraction of every molecule of histidine or of glutamic acid is assimilated as material of the composition $2.5(CH_2O)$; this balance of oxidative

TABLE I

Oxidation of Histidine and of Glutamic Acid by Histidine-Grown Cell Suspensions of A. aerogenes

Each Warburg vessel contained 0.75 ml. of resting cells suspended in 0.067 M phosphate buffer of pH 6 (optical density, measured in a Coleman spectrophotometer, model 14, at 590 mμ, about 0.1) and 0.25 ml. of water containing 10 μmoles of substrate. The results are corrected for endogenous respiration. At the time indicated the cells were removed by centrifugation, and the supernatant fluid was analyzed.

Experiment No.	1	2	3	4
Substrate	Histidine	Histidine	Histidine	Glutamic acid
Time, *min.*	10	40	60	110
	Δ *μmoles*	Δ *μmoles*	Δ *μmoles*	Δ *μmoles*
Substrate	−5.5	−10.0	−10.0	−10.0
O_2			−23.0	−25.0
CO_2			+26.4	+26.5
Urocanic acid	+0.3	0		
Ammonia, alkali-labile	+3.8	+9.2	+9.5	+0.7
Formic acid, alkali-labile	+3.5	+8.0		
Glutamic acid, free	+1.1	0		
Ammonia, free	+8.1	+14.3	+16.8	+6.8
Formic acid, free	+0.5	+1.3		

assimilation and degradation is similar to that observed when other carbon compounds are oxidized by *A. aerogenes* (17, 18). In addition, about 0.3 molecule of NH_3 seems to be taken up by the cells for every molecule of histidine or glutamic acid oxidized. The remainder of the histidine molecule accumulates in the suspending medium as NH_3 (1.7 moles per mole of histidine) and equimolar amounts of alkali-labile NH_3 and alkali-labile formic acid (0.95 mole per mole of histidine) (Table I). Histidine is not degraded by resting cells in the absence of oxygen unless a fermentable compound, such as glucose or pyruvic acid, is added to the reaction mixture.

Enzymatic Activities of Dried Cells—A. aerogenes grown on histidine and dried *in vacuo* over P_2O_5 degraded histidine under aerobic as well as anaerobic conditions. In the latter case glutamic acid was identified as one of the products. Urocanic acid was degraded in a similar manner, but at a more rapid rate than histidine. In resting cells on the other hand, the rate of histidine oxidation exceeded by far the rate of oxidation of urocanic acid. These dried cell preparations were also capable of catalyzing the production of glutamic acid from L-α-formamidinoglutaric acid. Similar preparations of glucose-grown cells did not attack histidine, urocanic acid, or L-α-formamidinoglutaric acid.

Degradation of Urocanic Acid by Cell-Free Extracts—The cell-free extracts used in these experiments were prepared from histidine-grown *A. aerogenes*, strain 50, and *P. fluorescens*, strain 6. The crude enzyme systems of either organism, when used in low concentration, degraded urocanic acid without the production of glutamic acid or of ammonia; the product of the reaction could be hydrolyzed by alkali to an equimolar mixture of glutamic acid and ammonia equivalent to about 75 per cent of the urocanic acid that had disappeared (Table II, Experiments 1 and 2). Paper chromatography in the butanol-formic acid solvent revealed the presence in the reaction mixture of a single compound which reacted with the ninhydrin reagent to produce a purple spot about 1 week after contact. The R_F value of this compound and its sluggish reaction with ninhydrin suggest its identity with L-α-formamidinoglutaric acid.

The action of more concentrated enzyme extracts of these two organisms on urocanic acid led to different end-products. The *Pseudomonas* preparation produced an equimolar mixture of glutamic acid, ammonia, and formic acid; under the conditions used the reaction did not go to completion, and about one-third of the urocanic acid was accounted for as an alkali-labile compound, presumably L-α-formamidinoglutaric acid, composed of equimolar quantities of glutamic acid, formic acid, and ammonia (Table II, Experiment 4). The *Aerobacter* preparation degraded urocanic acid to glutamic acid (1.03 moles per mole) and a compound hydrolyzable by alkali

to ammonia and formic acid (about 1 mole per mole of urocanic acid). The quantitative recovery of the degraded urocanic acid as free glutamic acid and alkali-labile formic acid and ammonia permits this alkali-labile product to be identified as formamide.

TABLE II

Degradation of Urocanic Acid by Cell-Free Extracts of Histidine-Grown A. aerogenes and P. fluorescens

The reaction mixture consisted of 1.0 ml. of 0.067 M phosphate buffer, pH 6.3, containing the amount of enzyme indicated (0.1 ml. of the *Aerobacter* extract contained 0.6 mg. of protein), and 10 μmoles of neutralized urocanic acid. The mixtures were incubated at 28° and at 37° in the case of the *Pseudomonas* and the *Aerobacter* extracts, respectively. Changing the pH to 7.0, or increasing the temperature to 37°, did not affect the balance of the products. The duration of Experiments 1 and 2 was 75 minutes and of Experiments 3 and 4, 110 minutes.

Experiment No.	1	2	3	4
Enzyme source	*A. aerogenes*	*P. fluorescens*	*A. aerogenes*	*P. fluorescens*
Enzyme, ml.	0.1	0.1	0.6	0.6
	Δ μmoles	Δ μmoles	Δ μmoles	Δ μmoles
Urocanic acid	−4.5	−8.6	−9.7	−10.0
Glutamic acid, alkali-labile	+3.2	+6.3	+0.4	+3.2
Ammonia, alkali-labile	+4.0	+6.3	+8.1	+3.5
Formic acid, alkali-labile			+10.4	+2.1
Glutamic acid, free	0	Trace	+10.0	+5.5
Ammonia, free	0	+0.5	+1.0	+6.1
Formic acid, free			0	+5.5

TABLE III

Degradation of Alkali-Labile Intermediate by Cell-Free Extracts of Histidine-Grown A. aerogenes and P. fluorescens

The experimental conditions correspond to those described in Table II.

Experiment No.	1	2	3
Enzyme source	*A. aerogenes*	*A. aerogenes*	*P. fluorescens*
Enzyme, ml.	0.6	0.6	0.6
Substrate source	Synthetic*	*P. fluorescens*†	*A. aerogenes*†
	Δ μmoles	Δ μmoles	Δ μmoles
Glutamic acid, alkali-labile	−8.6		
Ammonia, alkali-labile	+0.3	+0.2	−4.1
Glutamic acid, free	+8.6	+++‡	++‡
Ammonia, free	−0.4	+0.3	+3.5

* 10 μmoles of α-L-formamidinoglutaric acid.

† Prepared by the action of dilute enzyme solution (0.1 ml. per ml.) on 10 μmoles of urocanic acid.

‡ The presence of free glutamic acid (about 8 μmoles in Experiment 2 and 5 μmoles in Experiment 3) was demonstrated by paper chromatography.

Action of Cell-Free Extracts on L-*α-Formamidinoglutaric Acid, N-Formyl-*L-*glutamic Acid, and Formamide*—Synthetic L-α-formamidinoglutaric acid was hydrolyzed by concentrated *Aerobacter* extracts to the same products as urocanic acid, namely glutamic acid and formamide (Table III, Experiment 1). These compounds were also formed when the product of the action of *dilute* extracts of *P. fluorescens* on urocanic acid was treated with *concentrated* extracts of *A. aerogenes* (Table III, Experiment 2). On the other hand, the product of the action of the *dilute* extracts of *A. aerogenes* on urocanic acid was hydrolyzed by *concentrated Pseudomonas* extracts to free glutamic acid, ammonia, and presumably formic acid (Table III, Experiment 3). The same products were found by Borek and Waelsch (4) to result from the action of *Pseudomonas* extracts on L-α-formamidinoglutaric acid.

N-Formyl-L-glutamic acid was hydrolyzed by *Pseudomonas* extracts, with the production of free glutamic acid, as reported by Tabor and Mehler (19); *Aerobacter* extracts failed to catalyze this reaction.

Neither of the bacterial preparations was capable of hydrolyzing formamide.

DISCUSSION

The ability to dissimilate histidine is acquired by *A. aerogenes* through exposure to histidine (1). Adaptation to histidine endows the organism with the power to degrade not only histidine, but also glutamic acid and urocanic acid. Although the degradation of the latter compound is rapid in dried cells and cell-free extracts, it is very slow in intact cells, presumably because the intact cell wall is quite impermeable to this strongly acidic compound. The observation that histidine is degraded by dried cells, but not by intact cells in the absence of oxygen, suggests that energy, which may be obtained by endogenous respiration, is required for the transport of histidine across the intact cell wall; in the presence of a fermentable carbon compound which can serve as a source of energy, histidine is degraded by intact cells even in the absence of oxygen.

The "adaptive pattern" of histidine degradation in *A. aerogenes* is similar to the one observed by a group of Japanese workers in a strain of *Pseudomonas* (20) and implicates urocanic acid and glutamic acid as intermediates in the dissimilation of histidine. This view is confirmed by the results of the experiments comparing the oxidation of histidine and of glutamic acid by resting cells of *A. aerogenes* (Table I). The total uptake of oxygen and evolution of CO_2 were the same with the two substrates. In addition to the quantity of ammonia formed in the breakdown of glutamic acid, 1 mole of ammonia and 1 mole of a compound yielding equimolar amounts of ammonia and formic acid on alkaline hydrolysis, presum-

ably formamide, were produced from histidine. The fleeting appearance of small quantities of urocanic acid and of glutamic acid in the course of histidine degradation completes the evidence for the conversion of histidine via urocanic acid without the uptake of oxygen to formamide, ammonia, and glutamic acid, followed by the oxidative dissimilation of the last compound; a portion of the breakdown products is assimilated as material of the composition CH_2O.

The experiments in which cell-free extracts of histidine-grown cells were used provide information on the enzymatic steps between urocanic acid and glutamic acid (Tables II and III). Dilute enzyme preparations of *A. aerogenes* or of *P. fluorescens* converted urocanic acid to the same compound, L-α-formamidinoglutaric acid, isolated and identified previously by Borek and Waelsch as the final product of histidine degradation in cat liver extracts (4). Concentrated extracts of *A. aerogenes* hydrolyzed urocanic acid or L-α-formamidinoglutaric acid to a mixture of glutamic acid and formamide. Concentrated extracts of *P. fluorescens*, on the other

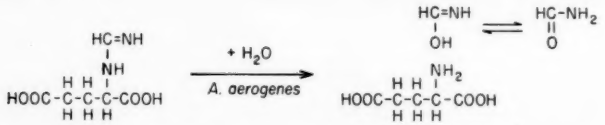


FIG. 1. Hydrolysis of L-α-formamidinoglutaric acid by *A. aerogenes*

hand, hydrolyze these compounds to glutamic acid, formic acid, and ammonia, as found previously by Tabor and his collaborators (3) and by Borek and Waelsch (4). The observation that *Pseudomonas* extracts can hydrolyze *N*-formyl-L-glutamic acid, but not formamide, confirms the results obtained by Tabor and Mehler (19), which show that in the extracts of *P. fluorescens* L-α-formamidinoglutaric acid is hydrolyzed to *N*-formyl-L-glutamic acid which is further split into formic acid and glutamic acid. The results of the present investigation indicate that *A. aerogenes* is unable to attack *N*-formyl-L-glutamic acid and converts L-α-formamidinoglutaric acid to glutamic acid and formamide as shown in Fig. 1.

The pathways of histidine degradation in cat liver, *P. fluorescens*, and *A. aerogenes* elucidated by the work of Borek and Waelsch (4), of Tabor and collaborators (2, 3, 5, 19), and by this investigation are illustrated in Fig. 2. The production of formamide as the final product of histidine metabolism seems to be the first instance of the appearance of formamide as a natural product.[1]

It is not clear why dilute extracts of either *A. aerogenes* or *P. fluorescens*

[1] The production of formamide from histidine has also been observed by one of us (H. R. B.) in extracts of histidine-grown *Pseudomonas aeruginosa*.

are incapable of hydrolyzing L-α-formamidinoglutaric acid. Either the instability of the enzymes responsible for these reactions or the lack of coenzyme may be the cause of this phenomenon. It is known that in tissues of higher organisms carbon 2 of the imidazole ring of histidine can serve as a source of formic acid for the biosynthesis of purines and of serine (21). In *A. aerogenes* formic acid is not a major product of histidine metabolism, and therefore the appearance of the ureido carbon of histidine in purines[2] may indicate the transfer of a single carbon unit directly from L-α-formamidinoglutaric acid or from formamide without the involvement of free formic acid. These problems are at present under investigation.

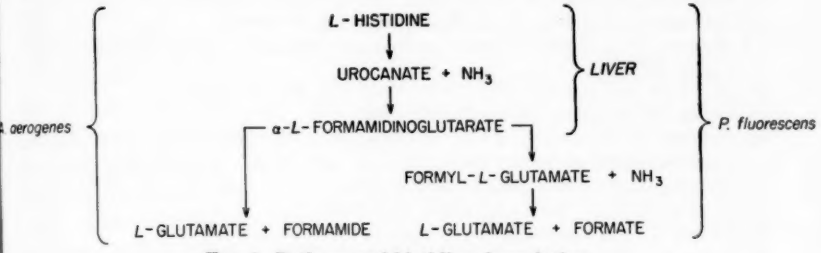


FIG. 2. Pathways of histidine degradation

SUMMARY

The degradation of L-histidine by histidine-grown *Aerobacter aerogenes* was studied in resting cell suspensions, vacuum-dried cells, and cell-free extracts. Exposure of the cells to histidine induces the formation of enzymes capable of attacking L-histidine, urocanic acid, L-α-formamidinoglutaric acid, and glutamic acid. Histidine is converted via urocanic acid and L-α-formamidinoglutaric acid to an equimolar mixture of ammonia, formamide, and glutamic acid; the last compound can be oxidized by resting cells to carbon dioxide, water, and ammonia. The pathways of histidine degradation of *Pseudomonas fluorescens* and of *A. aerogenes* were compared. In both organisms histidine is converted to L-α-formamidinoglutaric acid, which is also the final product of histidine metabolism in mammalian liver. Extracts of *P. fluorescens* hydrolyze this compound in two steps via *N*-formyl-L-glutamic acid to ammonia, formic acid, and glutamic acid. Extracts of *A. aerogenes* hydrolyze L-α-formamidinoglutaric acid to formamide and glutamic acid.

One of us (B. M.) is indebted to Miss Doris Karibian for excellent assistance.

[2] Bowser, H. R., and Magasanik, B., unpublished experiments.

BIBLIOGRAPHY

1. Magasanik, B., *J. Biol. Chem.*, **213**, 557 (1955).
2. Tabor, H., and Hayaishi, O., *J. Biol. Chem.*, **194**, 171 (1952).
3. Tabor, H., Mehler, A. H., Hayaishi, O., and White, J., *J. Biol. Chem.*, **196**, 121 (1952).
4. Borek, B. A., and Waelsch, H., *J. Biol. Chem.*, **205**, 459 (1953).
5. Mehler, A. H., and Tabor, H., *J. Biol. Chem.*, **201**, 775 (1953).
6. Edlbacher, S., and von Bidder, H., *Z. physiol. Chem.*, **276**, 126 (1942).
7. Ushiba, D., and Magasanik, B., *Proc. Soc. Exp. Biol. and Med.*, **80**, 626 (1952).
8. Sanger, F., and Tuppy, H., *Biochem. J.*, **49**, 463 (1951).
9. Jorpes, E., *Biochem. J.*, **26**, 1507 (1932).
10. Fowden, L., *Biochem. J.*, **48**, 327 (1951).
11. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948).
12. Awapara, J., and Seale, B., *J. Biol. Chem.*, **194**, 497 (1952).
13. Conway, E. J., Micro-diffusion analysis and volumetric error, London, 3rd edition (1950).
14. Grant, W. M., *Ind. and Eng. Chem., Anal. Ed.*, **18**, 729 (1946).
15. Grant, W. M., *Anal. Chem.*, **20**, 267 (1948).
16. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1946).
17. Magasanik, B., Brooke, M. S., and Karibian, D., *J. Bact.*, **66**, 611 (1953).
18. Magasanik, B., *J. Biol. Chem.*, **205**, 1019 (1953).
19. Tabor, H., and Mehler, A. H., *Federation Proc.*, **13**, 409 (1954).
20. Suda, M., Miyahara, I., Tomihata, K., and Kato, A., *Med. J. Osaka Univ.*, **3**, 115 (1952).
21. Sprinson, D. B., and Rittenberg, D., *J. Biol. Chem.*, **198**, 655 (1952).

STEROID METABOLISM IN GUINEA PIGS

I. METABOLISM OF CORTISOL IN VIVO*

By SHLOMO BURSTEIN and RALPH I. DORFMAN

With the Technical Assistance of Arline M. Tillotson and Paul R. Skogstrom

(From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts)

(Received for publication, August 25, 1954)

In previous studies (1–3) cortisol (hydrocortisone) was isolated and identified from the urine of the normal and the scorbutic guinea pig. However, paper partition chromatography of extracts of guinea pig urine (2) revealed that in addition to cortisol several other blue tetrazolium-reducing steroids were present. The possibility existed that some of these steroids were metabolites of endogenous cortisol, and it was believed that an understanding of the metabolic pathways operating in this animal would aid in the evaluation of corticosteroid excretion patterns under normal and pathological conditions. The study of the metabolism of cortisol in this animal therefore was undertaken.

Methods

Steroid Administration and Urine Extraction—Cortisol[1] was given orally to normal adult male guinea pigs. Urine was collected for 17 hours following steroid feeding, during which time more than 90 per cent of the metabolites containing the α-ketol side chain were excreted. The urine was extracted with ethyl acetate after adjustment to pH 6 by the addition of acetic acid to dissolve the precipitate present in guinea pig urine.

Paper Partition Chromatography—The crude extracts were fractionated by paper chromatography, as previously described, with the chloroform-formamide and the toluene-propylene glycol systems (4, 5). The individual zones were detected by the blue tetrazolium reagent and by examination of the chromatograms under ultraviolet light (Mineralight, Ultra-Violet Products, Inc., South Pasadena, California). The quantitative paper chromatographic procedure with ultraviolet spectrophotometry described previously (2) was employed for the quantitative determi-

* This work was supported in part by a research grant, NSF-G664, from the National Science Foundation, and by contract No. AT(30-1)-918 with the United States Atomic Energy Commission. A preliminary abstract has been presented at the 126th meeting of the American Chemical Society, New York, September 14, 1954.

[1] Thanks are due to Dr. K. Pfister for the gifts of cortisol, 6β,21-diacetoxy-17α-hydroxy-4-pregnene-3,11,20-trione, and 11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one.

XUM

nation of the urinary metabolites after administration of 20, 40, and 80 mg. of cortisol per animal.

Column Partition Chromatography—Column partition chromatography with acid-washed (6) Hyflo Super-Cel (Johns-Manville, New York) as the supporting phase was used (6, 7). A water-95 per cent ethanol solution 3:1 (volume per volume) was used as the stationary phase. The mobile phases consisted of organic solvents which were saturated with an equal volume of the stationary phase. The mobile phases included petroleum ether, toluene, benzene, benzene-methylene chloride 15:7, methylene chloride-benzene 15:7, methylene chloride containing 5 per cent butanol, methylene chloride-ethyl acetate 1:1, and ethyl acetate. All the solvents were redistilled, and no blank values as determined by weight were obtained. The fractionation procedure was followed by weighing. 40 ml. fractions were collected on an automatic fraction collector. A ratio of adsorbent to stationary phase 3:2 (gm. per ml.) was used.

Steroid Identification and Determination—The isolated steroids were identified by melting point determinations and by infra-red, visible, and ultraviolet spectrophotometry. All melting points were determined on the Kofler micro hot stage apparatus and are uncorrected. The infra-red spectra[2] were recorded on the Perkin-Elmer 12C model and were carried out on solid films deposited on sodium chloride, unless otherwise stated. The absorption coefficients in the ultraviolet were determined on the Beckman DU spectrophotometer and the spectra in sulfuric acid on the Cary recording 11MS spectrometer.

As the spectra in sulfuric acid of the isolated steroids characteristically change with time, they were recorded immediately (within 0.5 minute) and 120 minutes after dissolving the steroid in acid. The two spectra are characteristic of the steroid and adequately represent the change. The average recording time of a spectrum from 220 to 600 mμ was 10 minutes ± 1 minute.

EXPERIMENTAL

Isolation of Cortisol Urinary Metabolites—A total of 2 gm. of cortisol was fed to twenty guinea pigs, 100 mg. per guinea pig. The ethyl acetate extract of the urine was chromatographed in the chloroform-formamide system on twelve 15 cm. wide papers for 17 hours; Fig. 1 illustrates the chromatographic separation achieved. Fig. 2 shows the separation achieved by column partition chromatography of an extract of guinea pig urine following the feeding of 1 gm. of cortisol to ten guinea pigs, 100 mg. per animal. The description of steroid isolation follows.

[2] We are indebted to Dr. H. Rosenkrantz for advice in the interpretation of the infra-red spectra.

6β,11β,17α,21-Tetrahydroxy-4-pregnene-3,20-dione (6β-Hydroxycortisol) (I)—This steroid (as the acetate) was isolated from Zone I (Fig. 1) and from Fractions 61 to 66 (Fig. 2). A yellow-brown pigment consistently ran along with Zone I and, despite repeated paper chromatography in the chloroform-formamide system, no separation of the steroid from the pigment was achieved. As no purification of the bulk of the material was

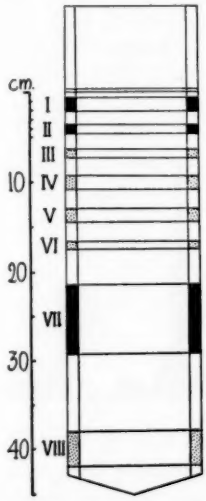


FIG. 1. Diagrammatic presentation of a paper chromatogram (run in the chloroform-formamide system for 17 hours) of the ethyl acetate extract of guinea pig urine after the oral administration of cortisol. Zones I to VIII were detected by examination under ultraviolet light. The reducing steroid zones were detected on strips cut along the sides of the paper by the blue tetrazolium color reaction, and the intensities of the observed blue colors are represented by the different shades. The major components of Zones I, IV, V, and VII were identified as 6β,11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione, 11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one, 11β,17α,20β,-21-tetrahydroxy-4-pregnen-3-one, and cortisol, respectively. The location of the zones is indicated on the scale in cm. from the starting line (the double line at 0 cm.).

possible by crystallization, the combined crystals and mother liquors comprising Zone I were acetylated at room temperature in the usual manner. The acetylated mixture was chromatographed in the toluene-propylene glycol system for 17 hours. The yellow-brown pigment stayed at the starting line. A major zone that reduced the blue tetrazolium reagent was observed under ultraviolet light at 9.7 to 14.2 cm. from the starting line. Three other zones in smaller concentrations were detected by ultraviolet scanning, but were not identified. The major blue tetrazolium-re-

XUM

ducing zone was rechromatographed twice on paper and crystallized from acetone, ethyl acetate, and finally from 95 per cent ethanol. Colorless needles were obtained which melted at 122–125°; $[\alpha]_D^{23}$ +82° (MeOH). Microanalysis indicated an elemental composition of $C_{25}H_{34}O_8 \cdot H_2O$, which

$C_{25}H_{36}O_9$. Calculated, C 62.48, H 7.55; found, C 62.10, H 7.44

is consistent with the compound, 6β,21-diacetoxy-11β,17α-dihydroxy-4-pregnene-3,20-dione monohydrate (I-ac.). The molecular extinction coefficient ($\lambda_{max.}$ 236 mμ; ε = 12,300) of the compound is in keeping with

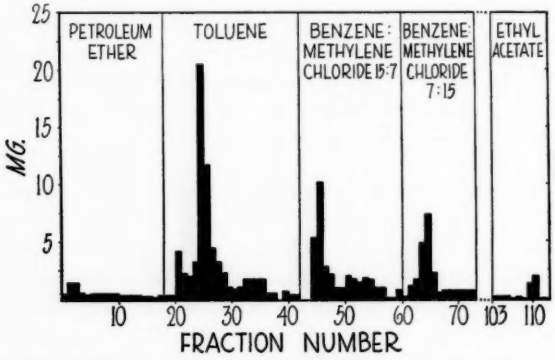
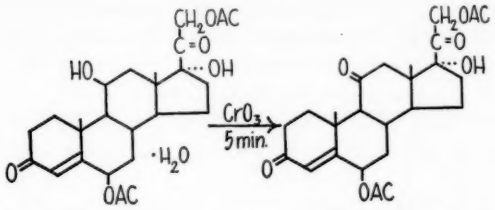


FIG. 2. Chromatographic pattern of a 145 mg. extract from urine collected from guinea pigs fed 1 gm. of cortisol. Chromatography on Hyflo Super-Cel partition column with water containing 25 per cent ethanol as the stationary phase. Elution with solvents saturated with the stationary phase as indicated. A total of 138 fractions of 40 ml. was collected. Fractions 74 to 77 were eluted with CH_2Cl_2, Fractions 78 to 86 with methylene chloride containing 5 per cent butanol, Fractions 87 to 102 with methylene chloride-ethyl acetate 1:1. The elution with ethyl acetate was continued to Fraction 138. No peaks were observed in these fractions.

the molecular extinction coefficients observed for other 6β-hydroxylated steroids (8). The infra-red spectrum (in a mull with paraffin oil) of this material is given in Fig. 3. The absorption spectrum of the isolated I-ac. in concentrated sulfuric acid is shown in Fig. 4. As can be seen from Fig. 4, the spectrum in sulfuric acid changes with time. However, the two extreme curves, immediately (within 0.5 minute) after (Curve 1) and 120 minutes after (Curve 5) dissolving in acid, represent two characteristic spectra of the steroid. Characteristic changes with time were also observed in the spectra of the other isolated steroids and are represented by the two extreme curves (see below). On dissolving I-ac. in sulfuric acid, a transient violet-purple color, but no green fluorescence, was obtained.



Identification of the isolated compound (I-ac.) was achieved by mild oxidation to 6β,21-diacetoxy-17α-hydroxy-4-pregnene-3,11,20-trione (6β-hydroxycortisone diacetate).

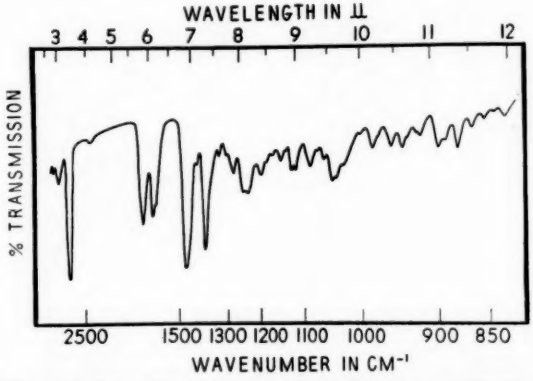


FIG. 3. Infra-red absorption spectrum of 6β,21-diacetoxy-11β,17α-dihydroxy-4-pregnene-3,20-dione monohydrate in a mull with paraffin oil.

The oxidation with chromic acid was carried out in a manner similar to that described by Reichstein (9) for the oxidation of cortisol acetate to cortisone acetate. To 2 mg. of I-ac. 0.3 ml. of 90 per cent acetic acid containing 3 mg. of CrO_3 was added and the mixture shaken at room temperature for 5 minutes. Following the addition of methanol, the solution was evaporated almost to dryness and extracted with ethyl acetate. The ethyl acetate was washed with water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Crystallization from 95 per cent ethanol afforded colorless crystals (rhombohedric rods) which melted at 225–233°. No depression in the melting point resulted by the admixture of an authentic sample of 6β-hydroxycortisone diacetate (m.p. 228–236°).[1] The infra-red spectrum and the absorption spectrum in sulfuric acid (Fig. 5) of the material were identical with those of the authentic sample. In addition, the isolated I-ac. was identical with the acetate of the incubation product

prepared by Hayano and Dorfman by the incubation of 6β,17α,21-trihydroxy-4-pregnene-3,20-dione with the 11β-hydroxylase system of beef adrenals (10).

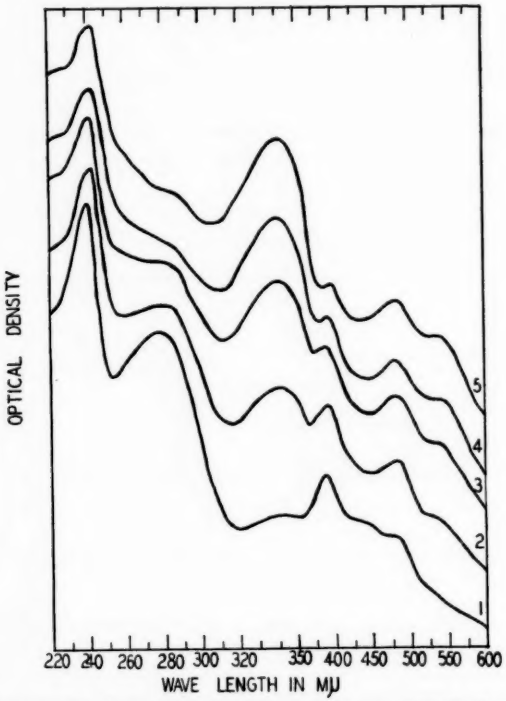


FIG. 4. Changes with time of the absorption spectrum of 6β,21-diacetoxy-11β,17α-dihydroxy-4-pregnene-3,20-dione monohydrate in sulfuric acid (approximately 150 γ in 3 ml.). The spectra are presented as recorded by the Cary 11MS recording spectrometer. The average recording time was 10 minutes ± 1 minute. Curves 1 to 5 were obtained within 0.5, 15, 30, 60, and 120 minutes, respectively, after dissolving the steroid in sulfuric acid. The curves were plotted one above the other for convenience. The optical densities at the maxima 240, 280, 340, 390, and 480 mμ were 1.77, 1.83; 1.26, 1.16; 0.54, 1.38; 0.60, 0.80; and 0.45, 0.73 for Curves 1 and 5, respectively. The optical density at the 550 mμ peak in Curve 5 was 0.59.

Hydrolysis of the diacetate monohydrate (I-ac.) to the free steroid (I) with alkali did not proceed smoothly. Herzig and Ehrenstein (8) have shown that 6β-acetoxy steroids isomerize in aqueous alcoholic alkaline media to the allopregnane-3,6-dione derivatives; smooth hydrolysis was shown to proceed in absolute ethanol under nitrogen. By using aqueous

methanolic $KHCO_3$, I-ac. gave only a poor yield of a material which had the mobility on paper of I. A greater yield of I was obtained when I-ac. was dissolved in oxygen-free ethanol and treated with 2.3 equivalents of ethanolic KOH. Paper chromatography in the chloroform-formamide system revealed the presence of four reducing components. The most polar major component was identical in mobility with I and showed a maximal

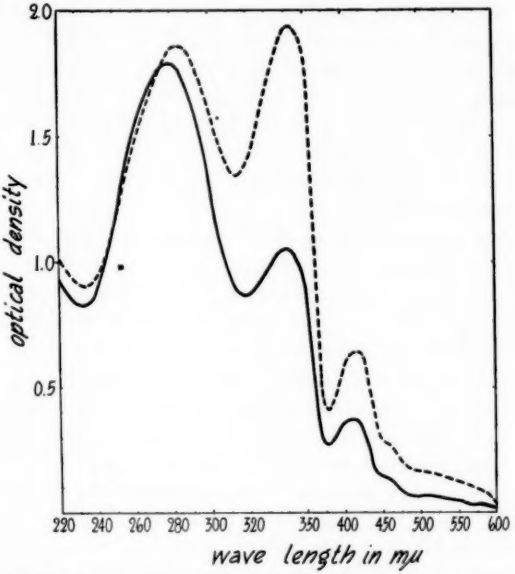


FIG. 5. Absorption spectrum of 6β,21-diacetoxy-17α-hydroxy-4-pregnene-3,11,20-trione in sulfuric acid (approximately 150 γ in 3 ml.), recorded by the Cary 11MS recording spectrometer. Average recording time, 10 minutes ± 1 minute. Solid line, spectrum obtained immediately (within 0.5 minute) after dissolving in sulfuric acid; broken line, 120 minutes after dissolving in acid.

absorption at 237 mμ. The material resisted crystallization, but the infra-red spectrum was identical with that of I prepared by Hayano and Dorfman (10). The other components of the hydrolysis could not be identified and probably are isomerization products. 6β-Hydroxycortisol was isolated in crystalline form from Fractions 61 to 66 after rechromatography on a Hyflo Super-Cel column (100 × 2.5 cm.). Crystallization of the major fraction from acetone gave colorless needles (m.p. 225–233°) which proved to be identical with I isolated by the hydrolysis of I-ac. The absorption spectrum of I in sulfuric acid was identical with that of I-ac.

Steroids IIa and IIb—Both of these steroids of unknown structure were isolated as their acetates from Zone II (Fig. 1), and only steroid IIb was isolated from Fractions 49 to 52 (Fig. 2). Zone II consisted of a mixture of at least two reducing steroids having similar mobilities, which were difficult to separate. The mixture was acetylated and chromatographed on a Hyflo Super-Cel partition column with benzene as the mobile phase. Two major reducing steroid acetates were eluted, and these will be referred to in order of increasing mobility, steroids IIa-ac. and IIb-ac. Steroid IIa-ac. was crystallized from 95 per cent ethanol to afford colorless prisms which melted at 223–230° (the larger crystals melted at 236°). The ultraviolet absorption spectrum in methanol had a maximum at 242 mμ ($E^{1\%}_{1\,cm.}$ 384). The infra-red spectrum had the following bands: hydroxyl at 3550, ester carbonyl at 1749, C-20 carbonyl at 1730, conjugated carbonyl at the unusually high frequency 1709, double bond at 1631, and acetate at 1230 and 1209 cm.$^{-1}$.

Steroid IIb-ac. crystallized in dense colorless needles which melted at 212–220°. The ultraviolet absorption spectrum in methanol had a maximum at 238 mμ ($E^{1\%}_{1\,cm.}$ 448). The infra-red spectrum had the following bands: hydroxyl at 3500, ester carbonyl at 1739, C-20 carbonyl at 1721, conjugated C-3 1669, Δ^4 double bond at 1631, and acetate at 1232 cm.$^{-1}$.

The absorption spectra in sulfuric acid of steroids IIa-ac. and IIb-ac. are shown in Fig. 6. Both steroids gave a green fluorescence in sulfuric acid. Steroids IIa-ac. and IIb-ac. have not been identified. Since Zone IV (Fig. 1) was identified as 11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one, it seems, in view of the structure-chromatographic mobility relationships (11), that steroids IIa and IIb may be $C_{21}O_6$ derivatives of cortisol.

The free steroid IIb was isolated from Fractions 49 to 52 (Fig. 2) after rechromatography on a Hyflo Super-Cel column. The major component crystallized readily by trituration with acetone, m.p. 234–238°. The infra-red spectrum had the following bands: hydroxyl at 3500, C-20 carbonyl at 1702, C-3 conjugated carbonyl at 1647, and Δ^4 double bond at 1625 cm.$^{-1}$.

Acetylation yielded colorless dense needles (from ethanol) which were identical (melting point, infra-red analysis, spectrum in sulfuric acid) with steroid IIb-ac. described above. Steroid IIa was not found after column chromatography.

11β,17α,20α,21-Tetrahydroxy-4-pregnen-3-one (IV)—This steroid was isolated from Zone IV (Fig. 1) and Fractions 44 to 48 (Fig. 2). Zone IV only slightly reduced the blue tetrazolium reagent and gave a strong orange-red dinitrophenylhydrazone. The eluate of this zone resisted crystallization and was chromatographed on a Hyflo Super-Cel column. The material came off the column with benzene containing 35 per cent methylene chloride and crystallized after trituration with neohexane and

acetone, m.p. 232–236°. The infra-red spectrum of this material was identical with that of 11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one (m.p. 239–243°).[1] The spectrum in sulfuric acid of the isolated compound

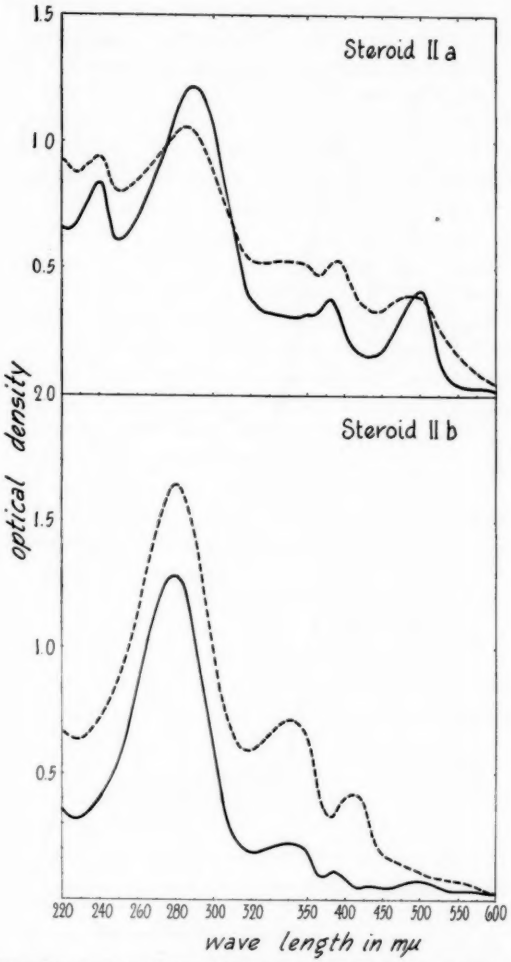


FIG. 6. Absorption spectra of steroids IIa-ac. and IIb-ac. in sulfuric acid (approximately 100 γ in 3 ml.), recorded by the Cary 11MS recording spectrometer. Average recording time, 10 minutes $\pm$ 1 minute. Solid line, spectrum obtained within 0.5 minute after dissolving in sulfuric acid; broken line, 120 minutes after dissolving in acid.

IV was identical with that of the authentic sample (see Fig. 7). A transient green fluorescence in sulfuric acid was observed. 11β,17α,20α,21-Tetrahydroxy-4-pregnen-3-one was isolated from Fractions 44 to 48 after

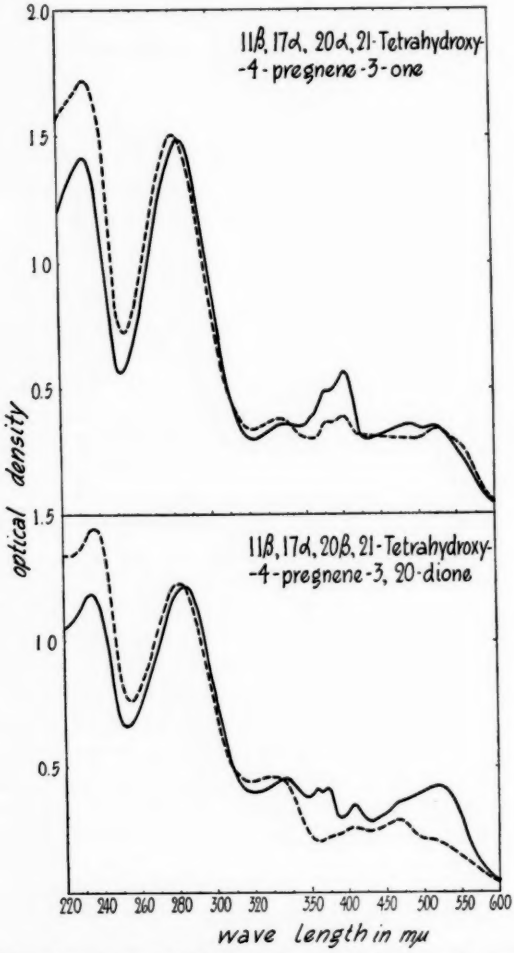


FIG. 7. Absorption spectra of 11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one (approximately 150 γ in 3 ml.) and 11β,17α,20β,21-tetrahydroxy-4-pregnen-3-one (approximately 100 γ in 3 ml.) in sulfuric acid, recorded by the Cary 11MS recording spectrometer. Average recording time, 10 minutes ± 1 minute. Solid line, spectrum obtained within 0.5 minute after dissolving in sulfuric acid; broken line, 120 minutes after dissolving in acid.

chromatography on a Hyflo Super-Cel column (100 × 2.5 cm.). Two major fractions were obtained which were identified as the 20α and 20β isomers.

11β,17α,20β,21-Tetrahydroxy-4-pregnen-3-one (V)—This steroid was isolated from Zone V (Fig. 1) and Fractions 44 to 48 (Fig. 2). Zone V gave the same color reactions as Zone IV, suggesting an intact Δ^4-3-keto system with a reduced side chain. Paper chromatography of Zone V in the toluene-propylene glycol system for 72 hours, followed by elution of the major steroid component and crystallization from acetone, resulted in crystalline material melting unsharply around 120°. The infra-red absorption spectrum and the spectrum in sulfuric acid (Fig. 7) of this material were identical with those of authentic 11β,17α,20β,21-tetrahydroxy-4-pregnen-3-one monohydrate. A transient fluorescence in sulfuric acid was observed. The acetate prepared in the usual manner melted at 210–216° (a change of crystal form from plates to needles occurred at 179°). The infra-red spectrum of the diacetate was identical with that of an authentic sample of 20β,21-diacetoxy-11β,17α-dihydroxy-4-pregnen-20-one.[3] Because of the similarity of the infra-red spectra of steroids IV and V, identification of these materials was difficult by this means. However, the absorption spectra in sulfuric acid were distinctly different (Fig. 7).

Cortisol—Cortisol was isolated from Zone VII (Fig. 1) and Fractions 23 to 29 (Fig. 2). Repeated paper chromatography in the toluene-propylene glycol system of the eluate of Zone VII revealed only a single reducing zone which was identified as cortisol by melting point determinations and infra-red spectrometry (2).

Zones III, VI, and VIII and additional steroids in the overflow were present in small amounts and were all α,β-unsaturated ketones as revealed by their ultraviolet absorption around 240 mμ. However, no conclusive identification of these zones was achieved. In addition, other fractions were eluted from the partition column, but were not identified with the exception of Fractions 107 to 110 which proved to be urea.

Determination of Cortisol Metabolites—By means of ultraviolet spectrophotometry and a quantitative paper chromatographic procedure described previously (2), the steroids in Zone I (6β-hydroxycortisol), Zone II (the mixture of steroids IIa and IIb), and Zone VII (cortisol) were quantitatively determined after administration of single doses of 20, 40, and 80 mg. of cortisol to individual guinea pigs. The results are presented in Table I. When an animal was used for more than one dosage level, an interval of 1 week elapsed between feedings. The steroids in Zones I, II, and VII were excreted in the urine in concentrations of 0.5 ± 0.1, 0.5 ± 0.10, and 2.7 per cent ± 0.3 per cent, respectively, of the 20 mg. of cortisol fed. The

[3] Generously supplied by Professor T. Reichstein.

administration of 40 mg. of cortisol resulted in the following percentage conversion into these urinary steroids: 0.8 ± 0.1, 0.4 ± 0.1, and 1.9 ± 0.4, respectively, and the administration of 80 mg. gave 0.9 ± 0.1, 0.7 ± 0.1, and 2.0 ± 0.6, respectively. The variation of recoveries from one experiment to another was so great that one is not justified in concluding that the

TABLE I

Quantitative Determination of Steroids in Zone I (6β-Hydroxycortisol), Zone II (Mixture of IIa and IIb), and Zone VII (Cortisol) by Ultraviolet Spectrophotometry

Cortisol administered	Guinea pig No.	Steroid recovered*		
		Zone I	Zone II	Zone VII
mg.		γ	γ	γ
20	1	90	50	510
	2	160	170	880
	3	50	80	316
	3	10	68	920
	3	240	50	750
	5			456
	5			730
	5	50	50	430
	7	50	110	400
	9	220	120	405
	10	100	110	200
	14	109	170	500
Mean ± s.e.†.....		108 ± 24	98 ± 15	541 ± 66
40	4	280	100	980
	6	420	200	600
	8	280	100	340
	11	360	220	960
	18	260	100	860
Mean ± s.e.....		320 ± 30	144 ± 27	748 ± 122
80	4	840	680	3460
	6	520	440	1600
	8	480	360	480
	11	800	440	1240
	18	800	680	1160
Mean ± s.e.....		688 ± 77	520 ± 67	1588 ± 502

* All values were calculated on the basis of the measured extinction coefficient of cortisol at λ = 240 mμ.

† Standard error.

excretion of the reducing steroid is significantly affected by the dose of cortisol administered. Table II includes the weight distribution in the various fractions following column chromatography. Of the crude crystalline ethyl acetate extract (145 mg.) applied to the column, 106 per cent was recovered in the 138 fractions collected (Fig. 2). A urine extract from the ten guinea pigs used prior to cortisol feeding weighed 20 mg. On the basis of crude crystalline weight (Table II), 4.6 per cent of the administered cortisol was recovered in the urine as such, 2.2 per cent as the 20α- and

TABLE II

Chromatographic Fractionation of Extract of Guinea Pig Urine (145 Mg.) after Oral Administration of 1 Gm. of Cortisol on Hyflo Super-Cel Column

Fraction No.	Weight	Major component
	mg.	
1–14	7.0	
15–22	9.4	
23–29	46.4	Cortisol
30–43	10.4	
44–48	21.5	11β,17α,20α,21-Tetrahydroxy-4-pregnen-3-one 11β,17α,20β,21-Tetrahydroxy-4-pregnen-3-one
49–52	6.3	Steroid IIb
53–57	5.3	Unidentified ring A-saturated steroid
58–60	0.9	
61–66	17.7	6β,11β,17α,21-Tetrahydroxy-4-pregnene-3,20-dione
67–106	21.0	
107–110	4.6	Urea
111–138	3.6	
Total.....	154.1	

20β-dihydro derivatives, 1.8 per cent as 6β-hydroxycortisol, and 0.63 per cent as steroid IIb. A similar chromatographic pattern was obtained in a second experiment, yielding 3.7 per cent cortisol, 2.1 per cent of the 20α- and 20β-dihydro derivatives, 1.5 per cent of the 6β-hydroxycortisol, and 0.4 per cent of steroid IIb.

DISCUSSION

It has been found that, following the administration of cortisol to normal adult male guinea pigs, a series of metabolites was excreted in the urine. 6β-Hydroxycortisol, 11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one, and

11β,17α,20β,21-tetrahydroxy-4-pregnen-3-one were isolated and identified as the major metabolites excreted in the free (unconjugated) form.

The isolation and identification of 6β-hydroxycortisol establishes the first instance of 6β-hydroxylation *in vivo*. The only 6-hydroxylated steroids thus far isolated from urine have been of the 6α configuration from human pregnancy urine (12). 6β-Hydroxylation has been demonstrated by the incubation of 11-deoxycorticosterone with hog adrenal mince (13), with homogenates of cow corpus luteum (14), and by the perfusion of progesterone through beef adrenals.[4] Recently, 6β-hydroxylation of 11-deoxycorticosterone, testosterone, and Δ^4-androstene-3,17-dione has been shown (by paper chromatography and color reactions) to occur in perfused rat liver (15). An efficient 6β-hydroxylating system has been shown to exist in microbiological preparations of *Rhizopus arrhizus* (16).

The biological significance of the 6β-hydroxylation in the guinea pig is not apparent. The isolation of 6β-hydroxycortisol from the urine of normal and scorbutic guinea pigs[5] shows that 6β-hydroxylation in this animal occurs also under physiological conditions. 6β-Hydroxylation *in vivo* is not unique for the guinea pig, since 6β-hydroxycortisol has now been isolated from human urine after administration of cortisol and from late human pregnancy urine (17).

Three minor zones were found in Zone I, less mobile than 6β-hydroxycortisol. One of the steroids in these zones may be the 6α isomer. However, the possibility that these zones may be artifacts should be considered in view of the isomerizations found by Herzig and Ehrenstein (8).

Steroids IIa and IIb seem to be (in respect to mobility) Δ^4-3-keto $C_{21}O_6$ steroids, the type and position of the additional oxygen being unknown, which would indicate that in addition to the 6β-hydroxylation another process for the introduction of an additional oxygen function is operating. Steroid IIa has been also isolated from the urine of normal and scorbutic guinea pigs.[5]

Another metabolic process established in this study is the reduction of cortisol to both the 20α- and 20β-dihydro derivatives.

SUMMARY

After administration of cortisol to guinea pigs, the following steroids were isolated and identified as the major metabolites present in the urine in the unconjugated form, in addition to unchanged cortisol: 6β,11β,17α,-21-tetrahydroxy-4-pregnene-3,20-dione (I), 11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one (IV), and 11β,17α,20β,21-tetrahydroxy-4-pregnen-3-one (V). Two unidentified steroids (IIa and IIb) were also isolated and seem

[4] Levy, H., unpublished data.

[5] Burstein, S., Dorfman, R. I., and Nadel, E. M., to be published.

to be (from mobility considerations) Δ^4-3-keto $C_{21}O_6$ derivatives of cortisol. Steroids I, IV + V, IIb, and cortisol, on the basis of crude crystalline weight determined by column partition chromatography, were excreted in concentrations of 1.77, 2.15, 0.63, and 4.64 per cent, respectively, of the fed cortisol.

Thanks are due to Dr. Frank Ungar for help in the preparation of the manuscript.

BIBLIOGRAPHY

1. Nadel, E. M., and Schneider, J. J., *Endocrinology*, **51**, 5 (1952).
2. Burstein, S., and Dorfman, R. I., *J. Biol. Chem.*, **206**, 607 (1954).
3. Burstein, S., Dorfman, R. I., and Nadel, E. M., *Federation Proc.*, **13**, 188 (1954).
4. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.*, **188**, 763 (1951).
5. Burstein, S., Savard, K., and Dorfman, R. I., *Endocrinology*, **53**, 88 (1953).
6. Butt, W. R., Morris, P., Morris, C. J. O. R., and Williams, D. C., *Biochem. J.*, **49**, 434 (1951).
7. Hagedüs, H., Tamm, C., and Reichstein, T., *Helv. chim. acta*, **36**, 357 (1953).
8. Herzig, P. T., and Ehrenstein, M., *J. Org. Chem.*, **16**, 1050 (1951).
9. Reichstein, T., *Helv. chim. acta*, **20**, 989 (1937).
10. Hayano, M., and Dorfman, R. I., *Arch. Biochem. and Biophys.*, **50**, 218 (1954).
11. Savard, K., *J. Biol. Chem.*, **202**, 457 (1953).
12. Lieberman, S., Fukushima, D. K., and Dobriner, K., *J. Biol. Chem.*, **182**, 299 (1950).
13. Haines, W. J., *Recent Progress Hormone Res.*, **7**, 282 (1952).
14. Hayano, M., Wiener, M., and Lindberg, M. C., *Federation Proc.*, **12**, 216 (1953).
15. Axelrod, L. R., and Miller, L. L., *Arch. Biochem. and Biophys.*, **49**, 248 (1954).
16. Peterson, D. H., Eppstein, S. H., Meister, P. D., Magerlein, B. J., Murray, H. C., Leigh, H. M., Weintraub, A., and Reineke, L. M., *J. Am. Chem. Soc.*, **75**, 412 (1953).
17. Burstein, S., Dorfman, R. I., and Nadel, E. M., *Arch. Biochem. and Biophys.*, **53**, 307 (1954).

C

By

(*From*

The
cortic
the m
substa
Bec
synthe
of a co
cortic
active
suppo
edge o
In t
hydro
and a
norma
steroid
treate

Ani
Labor
pigs, c
chased
derive
Medic
15 wee
land r
pany,
scorbu

* Su
Founda

XUM

CORTICOSTEROIDS IN THE URINE OF NORMAL AND SCORBUTIC GUINEA PIGS: ISOLATION AND QUANTITATIVE DETERMINATION*

By SHLOMO BURSTEIN, RALPH I. DORFMAN, AND ELI M. NADEL

(From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts and the National Cancer Institute, National Institutes of Health, Bethesda, Maryland)

(Received for publication, September 10, 1954)

The recent findings of a normal high excretion of formaldehydogenic corticoids in the urine of normal guinea pigs (1, 2) and the suggestion that the major corticosteroid excreted was cortisol (hydrocortisone) (3) were substantiated by the isolation of cortisol from the urine of this species (4).

Because ascorbic acid has been considered to be implicated in the biosynthesis of corticosteroids (5), it became important to study the effect of a complete dietary deficiency of ascorbic acid on the excretion of urinary corticosteroids in the guinea pig. The observed increase of biologically active cortisol-like material in the urine of scorbutic guinea pigs (3) receives support from the present communication, which also extends our knowledge of the corticosteroids excreted by this species.

In this paper we report the isolation of cortisol, 6β,11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione (6β-hydroxycortisol) (as the diacetate), and an as yet unidentified steroid (as the acetate) from the urine of both normal and scorbutic guinea pigs. Quantitative determination of these steroids was performed in controls, scorbutic animals, and scorbutic animals treated with corticotropin (ACTH).

Methods

Animals and Diet—All animals were obtained from the Animal Section, Laboratory Aids Branch, National Institutes of Health. Albino guinea pigs, descendants of Hartley stock animals, were used. They were purchased from Tumblebrook Farm, Brant Lake, New York, in 1950, and were derived from animals originally developed at the National Institute of Medical Research, England, by Sir Percival Hartley. Guinea pigs 10 to 15 weeks old, initially weighing between 295 and 345 gm., were fed Rockland rabbit ration, purchased through the Arcady Farms Milling Company, Chicago. This diet contains only traces of ascorbic acid and is scorbutigenic for guinea pigs (1, 3). Within 30 days after being placed

* Supported in part by research grant NSF-G664 from the National Science Foundation. An abstract has appeared (*Federation Proc.*, **13**, 188 (1954)).

on the unsupplemented diet, all animals were either moribund or had died with clinical, gross, and microscopic evidence of scurvy. The animals termed "scorbutic" were those exhibiting the characteristic lesions of advanced scurvy. The scorbutic guinea pigs had been on the deficient diet from 24 to 28 days. At the end of the experiment ten animals from scorbutic, underfed, control fasted, and normal control animals were decapitated, and representative tissues were histologically examined. The characteristic lesions of scurvy appeared in sections of the bones only in the scorbutic animals. When supplemented daily with 10 mg. of ascorbic acid given orally, the diet supported normal growth. All animals used as controls received this supplement daily.

Two sets of experiments were designed.

In *Experiment A* the urinary corticosteroid excretion pattern of scorbutic guinea pigs was compared with that of a group of control animals which were individually underfed and a group of fasted animals. The underfed and fasted animals received ascorbic acid daily. The diet of the underfed control animals was restricted during a 30 day period to an amount sufficient to sustain a weight loss approximating that which developed in the scorbutic animals. The weight loss of 23 per cent observed in the animals during the latter part of scurvy, after the animals had been on a deficient diet for 18 days or more, was not significantly different from the loss obtained in the control group. The fasted animals were not fed for 6 to 8 days, during which time they experienced a similar weight loss. Fasted animals were dead or moribund by the 10th day of fasting.

In *Experiment B* urine from normal controls receiving food *ad libitum* and ascorbic acid daily were compared with those of scorbutic animals and with those of scorbutic animals treated with ACTH. 20 units (i.u.) of ACTH (Acthar, Armour[1]) were administered in two equal doses, intraperitoneally and subcutaneously, to each animal.

Urine Collections—Urine was collected in all-glass metabolism cages.[2] Incorporated into each cage was an all-glass device and removable glass screen support which effectively separated urine from feces. Urine was collected from individual guinea pigs for 24 hours in Experiment A and for 17 hours in Experiment B. No food or water was administered during the period of urine collection.

Urine Extraction and Pooling of Extracts and Urine—As the purpose of this study was to determine, isolate, and identify corticosteroids containing the α-ketol side chain excreted in both scorbutic and control animals, it was necessary to pool the urines or extracts from similarly treated animals. In

[1] Thanks are due to Dr. E. Hays for the gift of the ACTH.

[2] The cages were devised for this study with the help of Mr. Frederick Highhouse, Instrument Section, Laboratory Aids Branch, National Institutes of Health.

Experiment A, urines from individual animals were extracted with ethyl acetate after acidification to pH 1 as previously described (4). Extracts from urine of similarly treated guinea pigs were then pooled into the following groups: Extract 1, underfed male controls; Extract 2, scorbutic males; Extract 3, underfed female controls; Extract 4, scorbutic females; and Extract 5, fasted males. The number of the 24 hour urine sample extracts pooled and the number of animals used are reported in Table I.

In Experiment B the urines were extracted at pH 6 and pooled into the following groups (Table I): Extract 1, normal male controls; Extract 2,

TABLE I

Sources of Urinary Extracts

Experiment A, 24 hr. urines				Experiment B, 17 hr. urines			
Extract No.	Type of guinea pig (days on scorbutigenic diet)	No. of guinea pigs used	No. of urines pooled	Extract No.	Type of guinea pig (days on scorbutigenic diet)	No. of guinea pigs used	No. of urines pooled
1	Underfed male controls	18	55	1	Normal male controls	40	88
2	Scorbutic (24–28) males	18	58	2	Scorbutic (24–26) males	51	120
3	Underfed female controls	8	21	3	Scorbutic (27) males during ACTH (20 i.u.) treatment	40	40
4	Scorbutic (24–28) females	8	21	4	Scorbutic (28) males after ACTH treatment	39	39
5	Fasted males	10	30				

male scorbutic animals; Extract 3, male scorbutic animals during ACTH treatment; Extract 4, male scorbutic animals 1 day after the ACTH treatment.

Fractionation by Paper Chromatography, Steroid Determination, Isolation, and Identification—The individual pooled urinary extracts were fractionated by paper partition chromatography with the chloroform-formamide and the toluene-propylene glycol systems (6). Only the steroids containing the reducing α-ketol side chain were investigated in this study. The appropriate steroid zones were quantitatively determined by ultraviolet spectrophotometry and by the phosphomolybdic acid reaction on the rechromatographed material, as previously described (4).

In Experiment B determinations by ultraviolet spectrophotometry were

XUM

also carried out on the rechromatographed acetylated steroids. Isolation was achieved by crystallization of the free steroids or of the acetates following rechromatography on paper. The isolated steroids were identified by melting point determinations, infra-red spectrometry, and their characteristic spectra in concentrated sulfuric acid. The melting points (uncorrected) were determined with the Kofler micro hot stage melting point apparatus. The infra-red spectra were recorded on solid films deposited on sodium chloride plates in the Perkin-Elmer 12C spectrometer. The absorption spectra in sulfuric acid were obtained with the Cary recording 11MS spectrometer. As the absorption spectra of the steroids isolated in sulfuric acid characteristically change with time, the spectra were obtained immediately (within 0.5 minute) and 2 hours after dissolving the steroids in sulfuric acid.[3]

EXPERIMENTAL

It was previously reported (2) that extracts of normal guinea pig urine contain in addition to cortisol, which was isolated and identified, several other blue tetrazolium-reducing steroids. Following chromatography of a guinea pig urine extract in the chloroform-formamide system for 17 hours, seven major zones were detected by scanning under ultraviolet light, three of which, Zones I, II, and VII (Fig. 1), contained the major reducing steroids. The chromatographic pattern was similar to that observed after feeding cortisol to normal guinea pigs.[3] Similar chromatographic patterns were observed after chromatography of the extracts of Experiments A and B.

The steroids of Zone I were determined by the Beckman DU spectrophotometer at 237 mμ, $E^{1\%}_{1\,cm.} = 270$, and Zones II and VII were determined at 240 mμ, $E^{1\%}_{1\,cm.} = 384$ and 413, respectively. These wave-lengths and absorption coefficients were used, since the major reducing steroids in Zones I, II, and VII were identified (see below) as 6β-hydroxycortisol, steroid IIa[3] (a steroid of unknown structure isolated from guinea pig urine after feeding cortisol), and cortisol, respectively.

Experiment A

Steroid Determination—The eluates of Zones I and II of the extracts of Experiment A were rechromatographed in the chloroform-formamide system, and those of Zone VII in the toluene-propylene glycol system for 72 hours. Table II presents the values for the steroids of Zones I, II, and VII of Extracts 1 to 5 as determined by the phosphomolybdic acid reaction. The data for Zones I and II represent only rough estimates, since these zones contained pigments which did not separate from the steroids even after rechromatography for 72 hours. Moreover, the major steroid com-

[3] Burstein, S., and Dorfman, R. I., to be published.

ponent of Zone I was identified as 6β-hydroxycortisol (see below). As this steroid is sensitive to acid, the values for Zone I are in doubt, since the urines in Experiment A were acidified to pH 1 before extraction. Little interfering pigment was present in Zone VII (which was identified as cortisol), and it was previously found that acidification to pH 1 does not

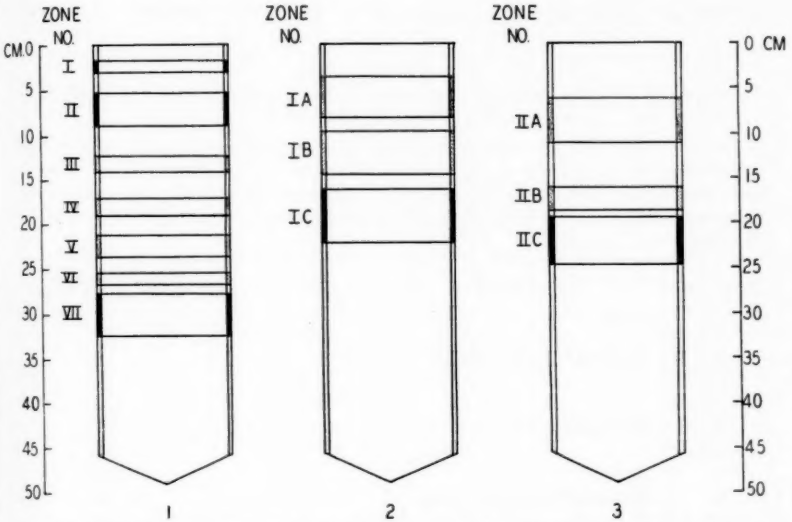


FIG. 1. Diagrammatic representation of paper chromatograms. The strips cut along the sides of the papers were developed by the blue tetrazolium color reaction, and the intensities of the observed blue colors are represented by different shading. All zones were also detected by observation under ultraviolet light. Paper chromatograms of (*1*) normal guinea pig urine extract run in the chloroform-formamide system for 17 hours; (*2*) acetylated eluate of Zone I (from chromatogram (*1*)) run in the toluene-propylene glycol system for 17 hours; (*3*) acetylated eluate of Zone II (from chromatogram(*1*)) run in the toluene-propylene glycol system for 12 hours.

alter the quantity of extractable cortisol in guinea pig urine (4). More accurate data for Zones I and II are given in Experiment B, in which the urines were extracted at pH 6.

Steroid Isolation and Identification. Zone I—Further purification of the steroids of Zone I for identification was achieved by acetylation and chromatography in the toluene-propylene glycol system for 17 hours. The major blue tetrazolium-reducing zone was eluted and the eluates crystallized. Insufficient material was present in Zone I of Extracts 1, 3, 4, and 5 to permit successful crystallization and identification by infra-red spec-

trometry. Zone I of Extract 2 yielded colorless needles which melted at 115–120°. The infra-red spectrum of the material was identical with that of authentic 6β-hydroxycortisol diacetate monohydrate (m.p. 122–125°). A violet-purple color was obtained in sulfuric acid. The spectrum in sulfuric acid is compared to that of authentic 6β-hydroxycortisol in Fig. 2. Absorption maxima of the major reducing component of Zone I of Extract 5 were at the same wave-lengths as for 6β-hydroxycortisol. However,

TABLE II

Analysis of Urinary Reducing Corticosteroids in Underfed Control and Scorbutic Guinea Pigs (Experiment A)

Determination by the phosphomolybdic acid reaction on eluates of the blue tetrazolium-reducing Zones I, II, and VII following rechromatography on paper of individual zones. Eluates of Zones I and II rechromatographed in the chloroform-formamide system and those of Zone VII in the toluene propylene-glycol system for 72 hours.

Extract No.	Type of animal	Zone No.	Steroids determined per guinea pig per 24 hrs.
			γ
1	Underfed male controls	I	21
		II	10
		VII	22
2	Scorbutic males	I	51
		II	53
		VII	122
3	Underfed female controls	I	11
		II	19
		VII	8
4	Scorbutic females	I	32
		II	63
		VII	104
5	Fasted males	I	30
		II	47
		VII	30

broadening of some bands occurred. An attempt to characterize the acetylated steroids of Zone I from Extracts 1, 3, and 4 by their absorption spectra in sulfuric acid was unsuccessful, owing to excessive background material which caused a broadening of the bands.

Zone II—The eluates of Zone II of Extracts 1 to 5 were acetylated and rechromatographed in the toluene-propylene glycol system for 12 hours. The major blue tetrazolium-reducing component was eluted. Only Extract 3 afforded crystalline material, melting at 170–180°. The infra-red spectrum of this material revealed the presence of impurities with resultant broadening and merging of some bands. However, it was possible to

identify the major component with steroid IIa (as the acetate), a urinary metabolite of unknown structure of cortisol in the normal guinea pig.[3] A green fluorescence in sulfuric acid was obtained; the absorption spectrum in sulfuric acid compared to that of steroid IIa is presented in Fig. 3.

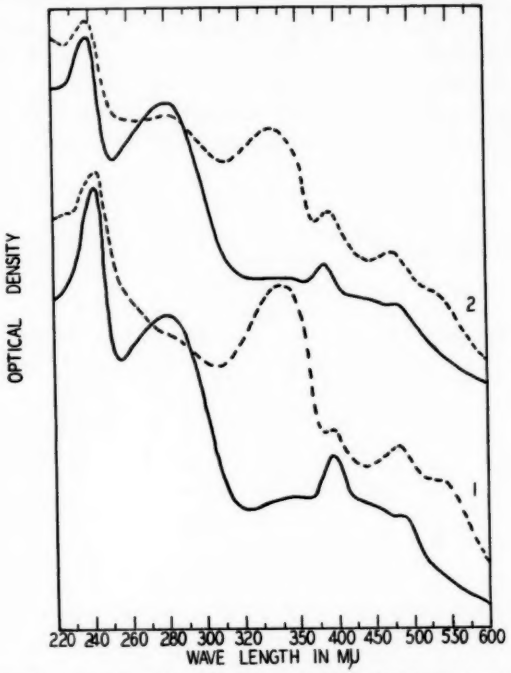


FIG. 2. Absorption spectrum in sulfuric acid of the isolated steroid from paper chromatogram Zone I of urine extract from scorbutic male guinea pigs (Experiment A, Extract 2) (Curve 2) compared to that of authentic 6β-hydroxycortisol diacetate monohydrate (Curve 1). Spectra recorded by the Cary 11MS recording spectrometer. Average recording time, 10 minutes ± 1 minute. Solid line, spectrum obtained immediately (within 0.5 minute) after dissolving in sulfuric acid; broken line, 120 minutes after dissolving in acid.

The acetates of Zone II of Extracts 4 and 5 gave absorption spectra in sulfuric acid with absorption maxima at the same wave-lengths as for steroid IIa. However, broadening of the bands resulted from the considerable background material present. Background material in Zone II of Extracts 1 and 3 made further characterization impossible.

Zone VII—Zone VII had the mobility of cortisol. Crystalline material was obtained from Zone VII of Extracts 1, 2, 4, and 5 which was con-

clusively identified as cortisol in the manner previously described (4). Insufficient material for infra-red spectrometry was present in Zone VII of Extract 3 to permit conclusive identification.

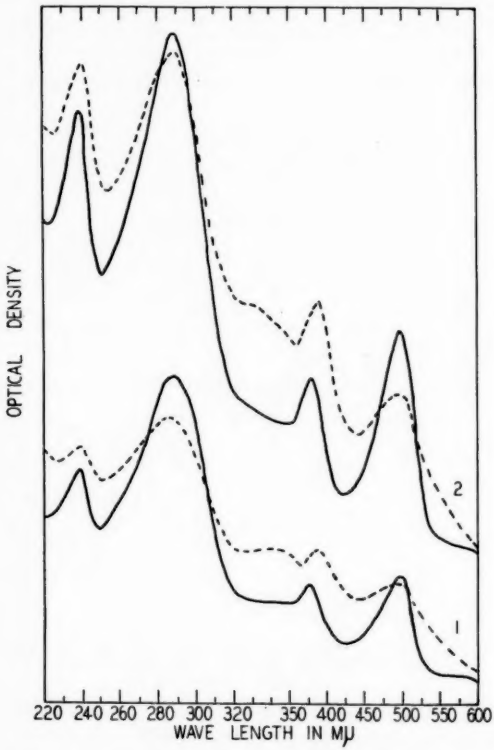


FIG. 3. Absorption spectrum in sulfuric acid of the isolated steroid from paper chromatogram Zone II of urine extract from scorbutic male guinea pigs (Experiment A, Extract 2) (Curve 2) compared to that of steroid IIa (isolated from normal guinea pig urine after feeding cortisol) (Curve 1). Spectra recorded by the Cary 11MS recording spectrometer. Average recording time, 10 minutes ± 1 minute. Solid line, spectrum obtained immediately (within 0.5 minute) after dissolving in sulfuric acid; broken line, 120 minutes after dissolving in acid.

Experiment B

The chromatographic pattern of the urinary extracts of Experiment B in the chloroform-formamide system was similar to that obtained in Experiment A (Fig. 1).

Steroid Determination—Table III presents results obtained by ultraviolet spectrophotometry and the phosphomolybdic acid reaction on the eluates of Zones I, II, and VII following chromatography of the Extracts 1 to 4. As in Experiment A, because of the presence of pigment, the data for Zones I and II are rough values. However, good agreement between the values in Tables II and III was obtained. In order to separate the steroids in Zones I and II from the interfering pigments, the eluates of these zones were acetylated and the acetates chromatographed in the toluene-propylene glycol system for 12 hours. The pigment stayed at the starting line, and a separation into three blue tetrazolium-reducing components which con-

TABLE III

Analysis of Urinary Reducing Corticosteroids in Normal Control, Scorbutic, Scorbutic Treated with ACTH, and Scorbutic Guinea Pigs after Treatment with ACTH (Experiment B)

Determinations on eluates of the blue tetrazolium-reducing Zones I, II, and VII following chromatography in the chloroform-formamide system for 17 hours. The results are expressed in micrograms per guinea pig per 24 hours.

Zone No.	Extract 1 Normal control		Extract 2 Scorbutic (day 24–26)		Extract 3 Scorbutic (day 27) ACTH		Extract 4 Scorbutic (day 28) after ACTH	
	UV*	PM*	UV	PM	UV	PM	UV	PM
I	22.8	38.7	44.3	52.5	38.7	61.2	57.4	86.4
II	26.5	42.0	36.0	46.3	43.2	61.2	46.7	59.4
VII	26.5	21.0	79.2	81.2	113.4	147.5	87.5	95.9

* UV, determined by ultraviolet spectrophotometry; PM, by phosphomolybdic acid reaction.

tained an α,β-unsaturated ketone system resulted. The eluates of Zone VII were rechromatographed in toluene-propylene glycol for 72 hours. A diagrammatic representation of the chromatograms of the acetates of Zones I and II appears in Fig. 1. The values for components A, B, and C of Zones I and II, determined by ultraviolet spectrophotometry, are presented in Fig. 4. No pigments were seen in the detected zones. The steroids in Zones IC and IIC and the rechromatographed Zone VII had maxima at 237, 242, and 240 mμ, respectively.

Steroid Isolation and Identification—The component of Zone IC of Extract 1 was crystallized from ethanol (95 per cent) to yield colorless needles melting at 117–120°. The infra-red spectrum and the absorption spectrum in sulfuric acid were identical with those of 6β-hydroxycortisol diacetate monohydrate. In a similar fashion, the component of Zone IC was iso-

lated and identified as 6β-hydroxycortisol diacetate monohydrate from the pooled Zone IC components of Extracts 2 to 4.

The component of Zone IIC of Extract 1 was crystallized from methanol to yield colorless rods melting at 212–220°. The infra-red spectrum and the absorption spectrum in sulfuric acid were identical with those of steroid IIa, isolated after administration of cortisol to normal guinea pigs. In a similar manner the combined Zone IIC components of Extracts 2 to 4 were identified as steroid IIa. The steroid in Zone VII was identified in Extracts 1 to 4 as cortisol, as previously described (4). Components A and B of Zones I and II were not further characterized.

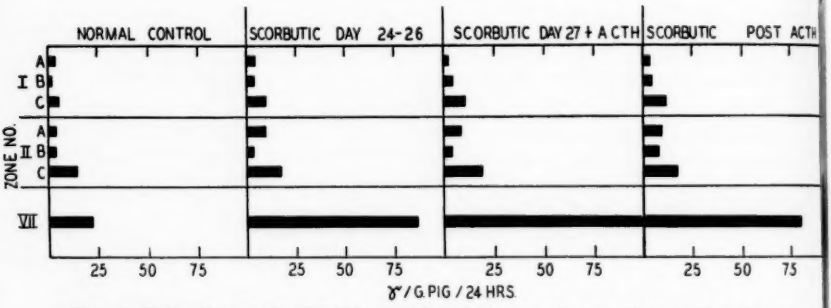


FIG. 4. Reducing corticosteroid excretion patterns of normal control and scorbutic guinea pigs with and without ACTH treatment. The steroids in Zones IC, IIC, and VII were identified as 6β-hydroxycortisol (as the diacetate monohydrate), steroid IIa (a urinary steroid of unknown structure, isolated after feeding cortisol to normal guinea pigs), and cortisol, respectively.

DISCUSSION

Within the past 3 years different techniques have been utilized to broaden our understanding of adrenocortical function in the scorbutic guinea pig (1, 3, 5, 7–13). In the present study corticosteroid excretion patterns in scorbutic animals were compared with those in normal controls, underfed controls, and fasted controls. In addition, the effect of ACTH on corticosteroid excretion in the scorbutic animal was studied. It is apparent from Table II that cortisol, which was isolated and identified from Zone VII, was excreted in the scorbutic animals in an increased concentration over that found in underfed male and female guinea pigs. No significant difference in the concentration of urinary cortisol was observed between the underfed controls and normal controls (compare Tables II and III). Fasting caused only a slight (possibly insignificant) increase above normal. Fig. 4 gives a diagrammatic presentation of the corticosteroid excretion patterns of normal control animals and scorbutic guinea pigs with and

without ACTH treatment. As can be seen, the steroids of Zone IC (6β-hydroxycortisol, as the diacetate monohydrate) and Zone VII (cortisol) were excreted in increased concentration in the scorbutic animals over that found in the normal controls. However, the data indicate that in scurvy the increase in the urinary concentration of 6β-hydroxycortisol over that found in the controls was 82 per cent, whereas the respective increase in cortisol was 300 per cent. No significant increase in the concentration of the steroid of Zone IIC was found in the scorbutic animals compared to the concentration present in the urine of the normal control animals. The steroid of Zone IIC (as the acetate) was isolated in crystalline form from all guinea pig urines studied and identified with a steroid of unknown structure isolated from normal guinea pig urine after feeding cortisol.[3] 6β-Hydroxycortisol and steroid IIa are thus regular urinary constituents, both in the normal and in the scorbutic guinea pig. 6β-Hydroxycortisol has now been isolated from human urine after feeding cortisol and from human pregnancy urine (14). 6β-Hydroxycortisol and steroid IIa are of a higher polarity than is cortisol and contain the α-ketol side chain and the Δ^4-3-keto system in ring A. The significance of the presence of these steroids in the urine of both normal and scorbutic guinea pigs is not apparent.

ACTH did not significantly increase the excretion of the corticosteroids in scorbutic guinea pigs. Previous studies (2) have shown that exogenous ACTH increases the concentration of urinary corticoids in the normal guinea pig. Administration of ACTH to normal control animals also caused an increase in the concentration of the urinary cortisol to a level similar to that found in the scorbutic animal.[4] This seems to indicate that the ineffectiveness of ACTH in the scorbutic animal may be due to an adrenal gland that is maximally stimulated by the stress of the vitamin deficiency and cannot be further stimulated by exogenous ACTH. Similar conclusions were arrived at by McKee and Walker (9), who found that ACTH did not cause a significant increase in the oxygen uptake of whole adrenal slices from scorbutic guinea pigs over that obtained with control scorbutic glands.

It is clear that the scorbutic guinea pig can synthesize cortisol despite the acute ascorbic acid deficiency. Increased quantities of material (11) giving a Porter-Silber reaction (15) and an increased quantity of cortisol have also been observed in the blood of scorbutic guinea pigs.[4] The presence of the increased quantities of cortisol in the urine and blood in scurvy may be the result of overproduction of cortisol by the hypertrophied adrenal cortex (5) owing to stress or to decreased metabolism of cortisol by specific tissues (3) or to a combination of both.

[4] Nadel, E. M., Burstein, S., and Dorfman, R. I., to be published.

SUMMARY

6β,11β,17α,21-Tetrahydroxy-4-pregnene-3,20-dione (as the diacetate) and cortisol were isolated in crystalline form and identified from the urine of normal control and scorbutic guinea pigs. In addition, a steroid of unknown structure (steroid IIa) was found to be excreted in both normal and scorbutic guinea pig urine. On the basis of ultraviolet spectrophotometry, 6β-hydroxycortisol was excreted in a concentration of 80 per cent and cortisol in a concentration of 300 per cent above the concentration in normal controls. Steroid IIa was not excreted in a significantly increased concentration over that found in normal controls. In scorbutic guinea pigs, ACTH treatment did not significantly increase the concentration of these urinary steroids over that present in untreated scorbutic animals.

We wish to express our appreciation to Miss Arline M. Tillotson, Mr. Leonard Saslaw, Mr. Bobby G. Young, and Mr. Arthur Hilgar for technical assistance in this study. Thanks are due to Mr. Paul Skogstrom for recording the infra-red and sulfuric acid spectra.

BIBLIOGRAPHY

1. Nadel, E. M., and Schneider, J. J., *J. Clin. Endocrinol.*, **11**, 791 (1951).
2. Burstein, S., *Endocrinology*, **50**, 412 (1952).
3. Nadel, E. M., and Schneider, J. J., *Endocrinology*, **51**, 5 (1952).
4. Burstein, S., and Dorfman, R. I., *J. Biol. Chem.*, **206**, 607 (1954).
5. Sayers, G., *Physiol. Rev.*, **30**, 241 (1950).
6. Burton, R. B., Zaffaroni, A., and Keutmann, E. H., *J. Biol. Chem.*, **188**, 763 (1951).
7. Morgan, A. F., *Vitamins and Hormones*, **9**, 161 (1951).
8. Eisenstein, A. B., and Shank, R. E., *J. Clin. Endocrinol.*, **11**, 791 (1951).
9. McKee, R. W., and Walker, J. K., *Science*, **118**, 133 (1953).
10. Clayton, B. E., and Prunty, F. T. G., *J. Endocrinol.*, **9**, 370 (1953).
11. Done, A. K., Ely, R. S., Heischt, L. R., and Kelly, V. C., *Proc. Soc. Exp. Biol. and Med.*, **83**, 722 (1953).
12. Becker, R. R., Burch, H. B., Salomon, L. L., Venkitasubramanian, T. A., and King, C. G., *J. Am. Chem. Soc.*, **75**, 2020 (1953).
13. Oesterling, M. J., and Long, C. N. H., *Science*, **113**, 241 (1951).
14. Burstein, S., Dorfman, R. I., and Nadel, E. M., *Arch. Biochem. and Biophys.*, **53**, 307 (1954).
15. Porter, C. C., and Silber, R. H., *J. Biol. Chem.*, **185**, 201 (1950).

ACTION OF THE NATURALLY OCCURRING TRYPSIN INHIBITORS AGAINST CHYMOTRYPSINS α AND B*

By FENG CHI WU and M. LASKOWSKI

(From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisconsin)

(Received for publication, September 23, 1954)

Kunitz and Northrop (2) were the first to observe that their crystalline pancreatic trypsin inhibitor also inhibited α-chymotrypsin, but "to a less marked extent." Several years later Kunitz crystallized soy bean trypsin inhibitor (3). He found it also inhibited α-chymotrypsin (4). In contrast to the reaction with trypsin which led to a formation of a stoichiometric complex, the soy bean inhibitor reacted with α-chymotrypsin by forming a highly dissociable complex.

Since several other trypsin inhibitors are now available in either crystalline or highly purified form, it was deemed advisable to investigate systematically the action of known trypsin inhibitors against chymotrypsins α and B, with the hope that such a study will help to characterize both the inhibitors and the enzymes and help to elucidate the nature of enzyme inhibitor interaction.

Methods

Crystalline trypsin, crystalline α-chymotrypsin, crystalline pancreatic trypsin inhibitor, and crystalline pancreatic trypsin inhibitor-trypsin complex were prepared by the method of Kunitz and Northrop (2), with the modification of McDonald and Kunitz (5) for the activation and crystallization of trypsin. Diisopropyl fluorophosphate-(DFP)-treated α-chymotrypsin and DFP-treated trypsin were gifts from Dr. E. F. Jansen, to whom we express our gratitude. Crystalline chymotrypsin B was prepared from crystalline zymogen (6, 7), as previously described (8).

Crystalline trypsin inhibitor from colostrum was prepared by the previously described method (9, 10). Crystalline soy bean inhibitor was purchased from the Worthington Biochemical Corporation. Crystalline soy bean complex was prepared according to Kunitz (11). Crystalline inhibitor from pancreas, described by Kazal, Spicer, and Brahinsky (12), to which we refer as Kazal's inhibitor, was a gift from Dr. L. A. Kazal, to whom we express our gratitude. Partially purified blood plasma inhibitor

* Aided by a research grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service. A preliminary report has been published (1).

was prepared according to Peanasky and Laskowski (13); the ovomucoid was prepared according to Fredericq and Deutsch (14).

The solutions of enzymes and inhibitors of desirable concentrations were prepared on the basis of optical density measurements. The concentration (mg. per ml.) was calculated by multiplying $E_{280}^{1\text{ cm.}}$ of the protein solution in acid medium by the respective factor. The optical factors for both chymotrypsins were reinvestigated on several different preparations. 0.495 was found for α-chymotrypsin, which was considered to be a good confirmation of the value of Kunitz (4) of 0.500, and the new factor for chymotrypsin B was established as 0.538, and for chymotrypsinogen B as 0.546. The optical factors for the inhibitors used in the present work were the same as previously reported (10). The optical factor for trypsin, as determined on DFP-treated trypsin, was 0.670. The factor 0.815 was used for soy bean inhibitor-trypsin complex.

The mixture of inhibitor and enzyme was allowed to equilibrate for at least 15 minutes before the substrate was added. This time interval was apparently sufficient, since longer exposures produced no change in the final equilibrium. No attempts were made to determine the minimal exposure time necessary to establish the equilibrium between the inhibitor and chymotrypsin.

Activity was determined either by the spectrophotometric method of Kunitz (4) on casein[1] or by the manometric method of Parks and Plaut (16) on L-phenylalanine ethyl ester hydrochloride[2] (PhEE). Under the conditions of assay, the ester alone had a weak but detectable rate of hydrolysis. All experimental values were corrected for the value of this blank. With the manometric technique a linear relationship between the initial rate of reaction (microliters of CO_2 liberated per minute) and the concentration of enzyme was observed for both chymotrypsins (Fig. 1). With PhEE, chymotrypsin B was found to be more active than α-chymotrypsin. Calcium was routinely added to attain the final concentration of 0.003 M. It was previously established that this amount of calcium increased the initial rate of the reaction by approximately 17 per cent.

In the spectrophotometric method of Kunitz (4), the solution of casein was made in 0.1 M borate buffer, pH 8.0, instead of 0.1 M phosphate buffer, and Ca was added to the system to attain the final concentration of 0.005 M. The activity curves thus obtained are represented in Fig. 2. In agreement with the previous findings (8), chymotrypsin B was less active than α-chymotrypsin with casein as substrate.[3]

[1] Prepared according to Dunn, M. S. (15).

[2] Purchased from Mann Research Laboratories, Inc., New York.

[3] Recent unpublished experiments indicate that the ratio between the activities of the two chymotrypsins is influenced by calcium concentration in the medium.

In systems containing inhibitors, the residual activity of chymotrypsins was determined by either of the two methods. When the casein method was used, the residual enzymatic activity was read from the standard curve (Fig. 2), and the amount of the inhibited chymotrypsin was calculated by the difference between the original and the recovered activity.

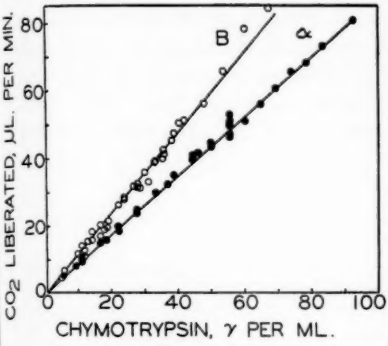


FIG. 1

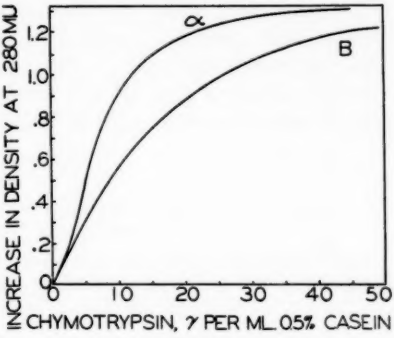


FIG. 2

FIG. 1. Manometric assay (16) for chymotrypsins α and B, at 30°, with PhEE as substrate. The initial rate of hydrolysis (microliters of CO_2 liberated per minute during the first 8 minute period) is plotted against enzyme concentration (micrograms of protein per ml. of reaction mixture). Final concentrations in the reaction mixture are 0.025 M PhEE, 0.042 M $NaHCO_3$, 0.003 M $CaCl_2$, 0.003 M borate buffer, pH 8.0, and indicated concentration of chymotrypsin. The atmosphere was 100 per cent carbon dioxide.

FIG. 2. Standard activity curves for chymotrypsins α and B, at 37°, with casein as substrate (4). The increase in optical density at 280 mμ after 20 minutes digestion is plotted against enzyme concentration (micrograms of protein per ml. of digestion mixture). Final concentrations in the digestion mixture are 0.5 per cent casein, 0.005 M $CaCl_2$, 0.05 M borate buffer, pH 8.0, and indicated concentration of chymotrypsin.

Results

Table I summarizes the results of the experiments in which the inhibitory powers of different naturally occurring trypsin inhibitors *versus* chymotrypsins α and B were investigated. All these experiments were performed by the casein digestion method (4).

As seen in Table I, the ability of different inhibitors to inhibit chymotrypsins varies widely. It is further seen that the two chymotrypsins studied are inhibited to a different extent by the same inhibitor. Generally, half inhibition of α-chymotrypsin is produced by a lesser concentration of a given inhibitor than the half inhibition of chymotrypsin B.

The only exception is soy bean inhibitor, which is more active toward chymotrypsin B.

Of six inhibitors tested, four reacted with chymotrypsins. Pancreatic inhibitor was most active against α-chymotrypsin and soy bean inhibitor against chymotrypsin B. The remaining two (ovomucoid and Kazal's inhibitor) failed to produce inhibition, even when 20 γ of inhibitor per 1 γ of enzyme were used. Further increase in the concentration of inhibitor would have probably resulted in the detection of inhibition, but such findings would be subject to criticism of obscuring the results by the non-specific effects of high protein concentration in the medium. Our finding in regard to ovomucoid is in agreement with the unpublished results

TABLE I

Concentrations of Inhibitors Required for 50 Per Cent Inhibition of Indicated Concentrations of Chymotrypsins at pH 8.0

Casein digestion method of Kunitz (4). Concentrations expressed in micrograms of protein per ml. of 0.5 per cent casein.

Inhibitor	α-Chymotrypsin		Chymotrypsin B	
	10 γ	25 γ	9.4 γ	23.5 γ
Pancreatic	2	4	150	
Kazal's (20:1)	Non-detectable		Non-detectable	
Soy bean	20	20	10	10
Colostrum	15	15	>1500	
Ovomucoid (20:1)	Non-detectable		Non-detectable	
Plasma, *units**	40	65	72.5	72.5

* A unit equals the amount of inhibitor capable of inhibiting 1 γ of trypsin (13).

of Lineweaver and Bean (17), who observed that "at least 25 times as much inhibitor was needed to inhibit any of ten other proteolytic enzymes studied as was required to inhibit trypsin." It does not, however, agree with the earlier findings of Lineweaver and Murray (18). The discrepancy may be due to the presence of a chymotrypsin-inhibiting impurity in the latter preparation.

Further analysis of data of Table I indicates that the interaction between chymotrypsins and inhibitors is not facilitated by the attractions between opposite net charges on the inhibitor and on the chymotrypsin molecules (19). The soy bean inhibitor with the isoelectric point of 4.5 (4) interacts more strongly with similarly charged chymotrypsin B, isoelectric at pH 4.7 (20), than with the oppositely charged α-chymotrypsin, isoelectric around pH 8.1 (21, 20). Analogous phenomenon has been observed with pancreatic inhibitor and α-chymotrypsin. A lower concentration of pancreatic

inhibitor with the isoelectric point >8.7 (22, 10) is required to cause half inhibition of the similarly charged α-chymotrypsin than is required for half inhibition of the oppositely charged chymotrypsin B.

All four active inhibitors formed with chymotrypsins dissociable complexes similar to the soy bean α-chymotrypsin complex previously described by Kunitz (4). Typical examples of such experiments are represented in Figs. 3 and 4. In these experiments a system composed of colostrum inhibitor and α-chymotrypsin was studied by the two different methods. The shape of the curves and the value for the half inhibition obtained by

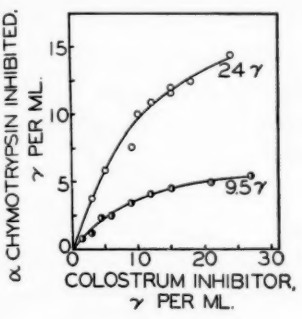


FIG. 3

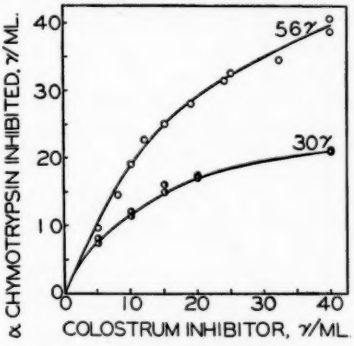


FIG. 4

FIG. 3. Effect of colostrum inhibitor on the digestion of casein by α-chymotrypsin. Concentration of α-chymotrypsin is indicated below the curve, concentration of colostrum inhibitor on the abscissa. Other conditions are as described in Fig. 2.

FIG. 4. Effect of colostrum inhibitor on the hydrolysis of PhEE by α-chymotrypsin. Concentration of α-chymotrypsin is indicated above the curve, concentration of colostrum inhibitor on the abscissa. Other conditions are as described in Fig. 1.

the two methods were similar, and were independent of the substrate used.

Different pairs of reactants formed complexes exhibiting a widely varied degree of dissociation. In the majority of cases, the value for half inhibition was identical for the two levels of the enzyme concentrations studied (Table I). However, some exceptions were noted.

Two interesting anomalies have been observed (Table I), the system composed of α-chymotrypsin and pancreatic inhibitor and the system composed of chymotrypsin B and soy bean inhibitor. The anomalous behavior becomes apparent when the results are calculated on the molecular basis. If the molecular weight of α-chymotrypsin is taken as 22,500 (23, 7) and that of pancreatic inhibitor as 9000 (22),[4] the maximal inhibitory activity expected from the uniunimolecular reaction would be 22,500:

[4] Evidence for this assumption has been recently discussed (19).

9000 = 2.5 γ of α-chymotrypsin inhibited per 1 γ of pancreatic inhibitor. As seen from Table I, half inhibition of 10 γ per ml. of enzyme is obtained at concentrations of inhibitor of 2 γ per ml. (inhibition ratio, 5:2 = 2.5). At concentration of α-chymotrypsin of 25 γ per ml., the half inhibition is obtained with 4 γ per ml. of inhibitor (inhibition ratio, 12.5:4 = 3.1).

Similar experiments were made with PhEE as substrate, at three different levels of α-chymotrypsin and variable concentrations of pancreatic inhibitor. The results are presented in Fig. 5. It is evident that the

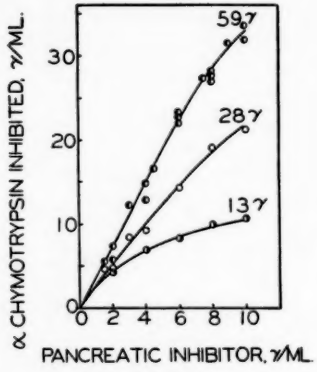


FIG. 5

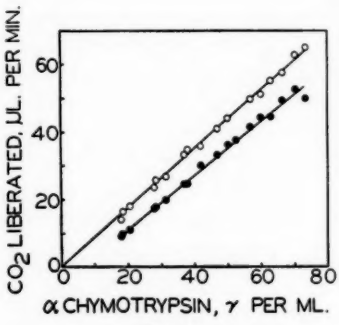


FIG. 6

FIG. 5. Effect of pancreatic inhibitor on the hydrolysis of PhEE by α-chymotrypsin. Concentration of chymotrypsin is indicated above the curve, concentration of pancreatic inhibitor on the abscissa. Other conditions are as described in Fig. 1.

FIG. 6. Effect of α-chymotrypsin concentration on the extent of inhibition of PhEE hydrolysis by pancreatic inhibitor. Concentration of α-chymotrypsin is indicated on the abscissa. ○, no inhibitor; ●, 3 γ of pancreatic inhibitor per ml. of the reaction mixture; other conditions are as described in Fig. 1.

amount of α-chymotrypsin inhibited per microgram of inhibitor increases with the increasing concentration of enzyme. At high concentration of enzyme (59 γ per ml.), about 4 γ of α-chymotrypsin are inhibited per 1 γ of inhibitor.

Fig. 6 gives the results of the experiments in which the concentration of α-chymotrypsin was varied, while the concentration of the pancreatic inhibitor was kept constant. The line representing the control experiments containing no inhibitor is also reproduced. The addition of inhibitor resulted not only in the change of the intercept, but also in the change of the slope. The slope of the line representing the experiments containing the inhibitor is smaller than that of the control line, indicating that, at higher

concentrations of enzyme, more α-chymotrypsin is inhibited per microgram of inhibitor. Extrapolation of the experimental line gives an intercept of about 6 γ of α-chymotrypsin, inhibition ratio 6:3 = 2, while drawing a line parallel to the control through the highest experimental point would give an intercept of about 13 γ (inhibition ratio 13:3 = 4.2). These inhibition ratios, expressed in molar concentrations and recalculated on the basis of the assumed molecular weights, are presented in Table II.

Analogous experiments were performed on the system, composed of soy bean inhibitor and chymotrypsin B. Fig. 7 illustrates the results of the experiments in which the concentration of inhibitor was varied, and Fig. 8 the experiments in which the concentration of inhibitor remained constant. The extreme inhibition ratios of the latter experiment expressed in molar concentrations are presented in Table II.

TABLE II

Inhibition Ratios Expressed in Molar Concentrations

The following molecular weights were assumed: α-chymotrypsin, 22,500; chymotrypsin B, 22,500; pancreatic inhibitor, 9000; soy bean inhibitors, 20,000; soy bean inhibitor-trypsin complex, 44,000.

Systems	Enzyme concentration, 10^{-6} M	Inhibitor concentration, 10^{-6} M	Enzyme inhibited, 10^{-6} M	Molar ratio, enzyme to inhibitor
α-Chymotrypsin *vs.* Pancreatic inhibitor	0.84 2.93	0.33 0.33	0.35 0.52	1.1 1.6
Chymotrypsin B *vs.* Soy inhibitor	0.67 2.68	0.20 0.20	0.29 0.53	1.5 2.7
Chymotrypsin B *vs.* Soy complex	0.95 2.00	0.46 0.46	0.29 0.43	0.63 0.95

In an attempt to elucidate further the anomalous behavior of the two systems mentioned above, the complexes of the respective inhibitors with trypsin were prepared and analyzed for the inhibitory activity against chymotrypsins. No inhibitory activity could be detected in the pancreatic inhibitor-trypsin complex against α-chymotrypsin, even when the concentration of the complex was 1000 γ per ml. Similarly, the addition of 100-fold excess of DFP-treated α-chymotrypsin to a system containing α-chymotrypsin and pancreatic inhibitor failed to divert the inhibitor from the native α-chymotrypsin.

Contrary to the previous case, the soy bean inhibitor-trypsin complex exerted a measurable inhibitory activity *versus* chymotrypsin B (Fig. 9, and

Table II). The observed inhibitory activity of the complex could not have been due to a contamination of the preparation with the excess of the free

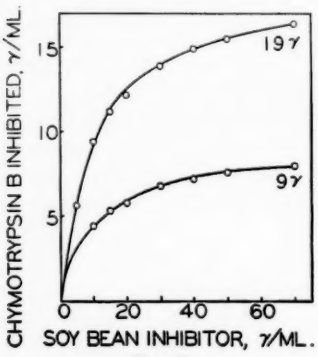


FIG. 7

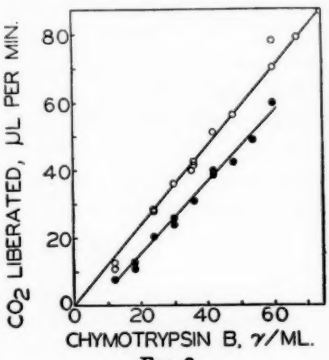


FIG. 8

FIG. 7. Effect of soy bean inhibitor on the digestion of casein by chymotrypsin B. Concentration of chymotrypsin B is indicated below the curve, concentration of soy bean inhibitor on the abscissa; other conditions are as described in Fig. 2.

FIG. 8. Effect of chymotrypsin B concentration on the extent of inhibition of PhEE hydrolysis by soy bean inhibitor. Concentration of chymotrypsin B is indicated on the abscissa; ○, no inhibitor; ●, 4 γ of soy bean inhibitor per ml. of the reaction mixture; other conditions are as described in Fig. 1.

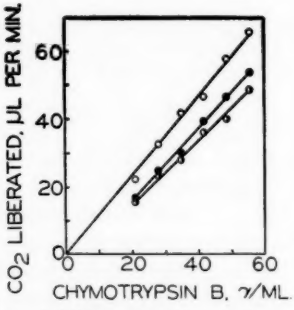


FIG. 9. Effect of soy bean inhibitor-trypsin complex on the hydrolysis of PhEE by chymotrypsin B. Concentration of chymotrypsin B is indicated on the abscissa; ○, no complex; ●, 20 γ of complex per ml. of reaction mixture; ◑, 20 γ of complex plus 20 γ of trypsin per ml. of reaction mixture; other conditions are as described in Fig. 1.

inhibitor. The complex was recrystallized six times and was analyzed for both tryptic and inhibitory activity by the casein method. No excess of the free inhibitor was found.

In order to minimize the dissociation of the complex, a large excess of trypsin was added to the system. The control experiment in which an equivalent amount of trypsin was used showed no significant increase in the rate of liberation of CO_2 beyond that of the blanks. The results, illustrated in Fig. 9, show that the addition of trypsin decreased the inhibition to some extent. The observed difference is interpreted as a measure of the contribution of the free inhibitor resulting from dissociation of the complex.

DISCUSSION

To our knowledge, the present work represents the first attempt at a systematic study of naturally occurring trypsin inhibitors as inhibitors of chymotrypsins. The finding that four of the six investigated inhibitors reacted with chymotrypsins would hardly be expected if six proteins were chosen at random and tested. This suggests that the spatial arrangements on or in proximity to the active center of trypsin and chymotrypsins are similar. It may be interesting to note that the two trypsin inhibitors which did not react with chymotrypsins are the two *temporary inhibitors* of trypsin (24–26).

The observed anomalies are not readily explicable without making rather unusual assumptions, which do not seem to be justified at present. This cautious attitude arises from an analysis of errors involved in calculating the molecular ratios of enzyme to inhibitor. The ratio is calculated on the basis of presently accepted molecular weights. A survey (19) of the reported molecular weights indicates that the values differ considerably with the method of determination and should be considered as probable rather than as certain. Our own determinations of concentrations are based on the optical factors. Changes in optical factors have been recently reported (27), and the optical factors for chymotrypsins, but not for the inhibitors, have been rechecked.

It seems, however, justifiable to consider the two observed anomalies as two separate cases, since they differ in one significant respect. The complex of trypsin with pancreatic inhibitor is inactive against α-chymotrypsin, whereas the complex of trypsin with soy bean inhibitor exerts inhibitory action *versus* chymotrypsin B.

Finally, attention must be called to the fact that our observations in respect to pancreatic and soy bean inhibitor confirm the observations of Kunitz and Northrop (2) and Kunitz (4), but are in contradiction to the results of Ravin, Bernstein, and Seligman (28), who found that the activity of 10 γ of crystalline α-chymotrypsin was unaffected by as much as 300 γ of soy bean inhibitor. The only plausible explanation for this discrepancy appears to be that the 20 per cent methanol used in the assay medium

of Ravin *et al.* (28) either denatured one of the reactants or prevented the reaction between α-chymotrypsin and the inhibitor.

We are greatly indebted to Dr. M. Laskowski, Jr., for many helpful suggestions and discussions.

SUMMARY

Several naturally occurring trypsin inhibitors were investigated as inhibitors for chymotrypsins α and B. None of the inhibitors formed a stoichiometric complex with either of the two chymotrypsins. With the exception of Kazal's inhibitor and ovomucoid, which were inactive against both chymotrypsins, all other trypsin inhibitors exhibited a variable degree of inhibitory activity against chymotrypsins. Half inhibition of α-chymotrypsin was attained with a lesser concentration of a given inhibitor than half inhibition of chymotrypsin B, with the exception of soy bean inhibitor, which was more active toward chymotrypsin B.

Two anomalies have been observed; namely, the systems composed (*a*) of pancreatic inhibitor and α-chymotrypsin, and (*b*) of soy bean inhibitor and chymotrypsin B. In these systems at high enzyme and low inhibitor concentrations, more than 1 molecule of the enzyme was inactivated by 1 molecule of the inhibitor.

BIBLIOGRAPHY

1. Wu, F. C., and Laskowski, M., *Federation Proc.*, **13,** 326 (1954).
2. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **19,** 991 (1936).
3. Kunitz, M., *J. Gen. Physiol.*, **29,** 149 (1946).
4. Kunitz, M., *J. Gen. Physiol.*, **30,** 291 (1947).
5. McDonald, M. R., and Kunitz, M., *J. Gen. Physiol.*, **29,** 155 (1946).
6. Laskowski, M., *J. Biol. Chem.*, **166,** 555 (1946).
7. Smith, E. L., Brown, D. M., and Laskowski, M., *J. Biol. Chem.*, **191,** 639 (1951).
8. Brown, K. D., Shupe, R. E., and Laskowski, M., *J. Biol. Chem.*, **173,** 99 (1948).
9. Laskowski, M., Jr., and Laskowski, M., *J. Biol. Chem.*, **190,** 563 (1951).
10. Laskowski, M., Jr., Mars, P. H., and Laskowski, M., *J. Biol. Chem.*, **198,** 745 (1952).
11. Kunitz, M., *J. Gen. Physiol.*, **30,** 311 (1947).
12. Kazal, L. A., Spicer, D. S., and Brahinsky, R. A., *J. Am. Chem. Soc.*, **70,** 3034 (1948).
13. Peanasky, R. J., and Laskowski, M., *J. Biol. Chem.*, **204,** 153 (1953).
14. Fredericq, E., and Deutsch, H. F., *J. Biol. Chem.*, **181,** 499 (1949).
15. Dunn, M. S., *Biochem. Preparations*, **1,** 22 (1949).
16. Parks, R. E., Jr., and Plaut, G. W. E., *J. Biol. Chem.*, **203,** 755 (1953).
17. Lineweaver, H., and Bean, R. S., quoted by Fraenkel-Conrat, H., Bean, R. S., and Lineweaver, H., *J. Biol. Chem.*, **177,** 385 (1949).
18. Lineweaver, H., and Murray, C. W., *J. Biol. Chem.*, **171,** 565 (1947).
19. Laskowski, M., and Laskowski, M., Jr., *Advances in Protein Chem.*, **9,** 203 (1954).

20. Kubacki, V., Brown, K. D., and Laskowski, M., *J. Biol. Chem.*, **180,** 73 (1949).
21. Anderson, E. A., and Alberty, R. A., *J. Phys. and Colloid Chem.*, **52,** 1345 (1948).
22. Green, N. M., and Work, E., *Biochem. J.*, **54,** 257 (1953).
23. Schwert, G. W., *J. Biol. Chem.*, **179,** 655 (1949).
24. Gorini, L., and Audrain, L., *Biochim. et biophys. acta*, **8,** 702 (1952).
25. Gorini, L., and Audrain L., *Biochim. et biophys. acta*, **10,** 570 (1953).
26. Laskowski, M., and Wu, F. C., *J. Biol. Chem.*, **204,** 797 (1953).
27. Green, N. M., and Neurath, H., *J. Biol. Chem.*, **204,** 379 (1953).
28. Ravin, H. A., Bernstein, P., and Seligman, A. M., *J. Biol. Chem.*, **208,** 1 (1954).

THE

TI

Evi
utiliz
reviev
have
group
(4–7),
ciency
of lab

Rat
acid,
inject
were
group
anima
isotop
and
labele
exper
In the
(Rats
ficien
obser
thesis

The
on th
such
of ch
A def
(10,
was

* F
† P
search

THE INFLUENCE OF VITAMIN B_{12} ON THE BIOSYNTHESIS OF THE METHYL GROUP OF CHOLINE FROM METHANOL

By WALTER G. VERLY* and WILLIAM J. CATHEY†

(From the Department of Biochemistry, Cornell University Medical College, New York, New York)

(Received for publication, September 27, 1954)

Evidence that vitamin B_{12} affects in some manner the biosynthesis or utilization of labile methyl groups in various organisms has been extensively reviewed (1–3). Studies of this effect with known labile methyl precursors have been of recent interest, and, since it has been shown that the methyl groups of choline can be derived from administered methanol in the rat (4–7), it seemed pertinent to investigate the effect of vitamin B_{12} deficiency on the availability of carbon from this source for the biosynthesis of labile methyl groups.

Rats deficient in vitamin B_{12} or doubly deficient in vitamin B_{12} and folic acid, prepared as described in the experimental section, were given a single injection of C^{14}-methanol approximately 3 weeks after weaning. They were sacrificed 8 hours after injection, and the radioactivity of the methyl groups of the isolated choline was determined. As shown in Table I, the animals deficient only in vitamin B_{12} (Rats 103 and 104) incorporated less isotope into the choline methyl group than the control animals (Rats 101 and 102) which received vitamin B_{12} 2 days before injection with the labeled methanol. In earlier experiments in which somewhat different experimental conditions were used, this difference was not detected (8). In the present work, animals deficient in folic acid as well as in vitamin B_{12} (Rats 304 and 203) appeared to incorporate less isotope than animals deficient in vitamin B_{12} alone (Rats 103 and 104). This is consistent with observations on the effect of folic acid and leucovorin on labile methyl synthesis from methanol already reported from this laboratory (8, 9).

The effect of a vitamin B_{12} deficiency or a folic acid deficiency in the rat on the extent of the incorporation of carbon from other labeled precursors such as C^{14}-formate, α-C^{14}-glycine, and β-C^{14}-serine into the methyl group of choline and methionine has been studied in other laboratories (10–15). A deficiency of vitamin B_{12} appears to decrease the utilization of formate (10, 11) and of the α-carbon of glycine (10–14). A deficiency in folic acid was likewise found to decrease the incorporation of these precursors into

* Fellow of the Belgian-American Educational Foundation.

† Postdoctoral Fellow of the Atomic Energy Commission under the National Research Council.

XUM

the methyl group of choline (10, 13). In contrast to their earlier finding (14) with respect to the β-carbon of serine, Arnstein and Neuberger (11) have more recently reported an increase in the incorporation of this precursor into the methyl group of choline and methionine on the administration of vitamin B_{12} to rats on a diet deficient in this factor. Stekol and coworkers, however, have reported that the utilization of this precursor for the synthesis of the methyl group of choline (13) and methionine (15) is not decreased in vitamin B_{12}-deficient rats, but that the synthesis of the methyl group of choline from this precursor is reduced in folic acid deficiency (13). Stekol, Anderson, Weiss, and Hsu (15) found that the synthesis of methionine from formate, the α-carbon of glycine, or the β-carbon of serine was not reduced in folic acid-deficient rats; the administration of leucovorin to such rats affected slightly the incorporation of the carbon from these precursors into the methyl group of methionine, and increased greatly the incorporation of this carbon into the methyl group of choline.

TABLE I

*Isotopic Content in Methyl Groups of Choline Isolated from Deficient and Control Animals Injected with C^{14}-Methanol**

Experimental group No.	Rat No.	Sex	Diet	Supplement	Trimethylamine chloroplatinate	
					Pt analysis†	C^{14} content, c.p.m. per mmole
I	101	♀	A	Vitamin B_{12}	37.0	13.6×10^4
	102	♂	"	" "	36.7	8.85×10^4
	103	♀	"	None	36.9	2.70×10^4
	104	♂	"	"	36.8	3.80×10^4
II	201	♂	B	Vitamin B_{12}	36.8	4.02×10^4
	202	♂	"	" "	37.1	4.60×10^4
	304	♂	"	None	37.2	1.97×10^4
	203	♂	"	"	37.2	0.72×10^4

* The C^{14} content of the administered methanol was 7.72×10^7 c.p.m. per mmole.
† Theoretical Pt content, 37.0.

In experiments *in vitro*, Sakami and Welch (16) found that the addition of vitamin B_{12} to a mixture containing homocysteine, C^{14}-formate, and liver slices from rats deficient in vitamin B_{12} did not increase the activity of the tetramethylammonium iodide obtained by hydriodic acid hydrolysis of the incubation mixture. On the other hand, the addition of folic acid to liver slices from folic acid-deficient rats increased the amount of methyl synthesis from formate.

EXPERIMENTAL

Two diets deficient in vitamin B_{12}, designated Diets A and B, were used in these experiments. Diet A contained the following per 100 gm. of diet: ethanol-extracted casein 25 gm., Osborne and Mendel salt mixture 4 gm., sucrose 65 gm., yeast 4 gm., water-soluble vitamin mixture[1] 1 gm., corn oil (Mazola) 1 gm., α-tocopherol acetate 4 mg., 2-methyl-1,4-naphthoquinone 0.1 mg., vitamin A 750 i.u., and vitamin D 125 i.u. Diet B was deficient in folic acid as well as in vitamin B_{12}. It differed from Diet A by the omission of folic acid and yeast, the addition of sulfasuxidine at a level of 2 per cent, and the increase in the sucrose level to 67 per cent.

Two groups of animals were used. The animals in Group I (Rats 101 to 104, Table I) were bred from a mother fed Diet A through the latter part of pregnancy and through lactation. The young were weaned 25 days following parturition and put on Diet A for 21 days. During this period each received four intraperitoneal injections of folic acid, the total dose being 1.7 mg. The control animals (Rats 101 and 102) were injected intraperitoneally with 10 γ of vitamin B_{12} 2 days before injection with 0.86 mmole of the labeled methanol. The animals in Group II were bred from mothers fed Diet B through gestation and lactation. The young were weaned 21 days after birth and were put on Diet B. Several of the animals died, and the survivors at about the 10th day following weaning lost weight over a period of several days but eventually resumed growth. These animals (Rats 201, 202, 203, and 304, Table I) were injected with 0.86 mmole of C^{14}-methanol 25 days after weaning. The control animals (Rats 201 and 202) received intraperitoneally 10 γ of vitamin B_{12} 4 days before and 1 day before the methanol injection. The radioactivity of the labeled methanol is given in Table I.

The animals in both groups were sacrificed 8 hours after the injection of the methanol, and choline was isolated from the carcasses as the chloroplatinate and degraded to trimethylamine as previously described (18). Platinum analyses on the chloroplatinates are recorded in Table I. The radioactivity of the injected methanol was assayed by combustion of a weighed aliquot of the aqueous solution in a current of oxygen and conversion of the carbon dioxide to barium carbonate. The C^{14} activity of the barium carbonate was determined with an end-window Geiger-Müller counter. The results were corrected for background and self-absorption. The samples of trimethylamine chloroplatinate were oxidized with the Van Slyke-Folch oxidizing mixture (19), and the C^{14} activity of the barium carbonate was determined as already indicated.

[1] The vitamin mixture was patterned after one described previously (17), the only changes involving the omission of vitamin B_{12} and an increase in the level of folic acid from 0.4 mg. to 1 mg. per gm. of vitamin mixture.

SUMMARY

Methyl synthesis from C^{14}-methanol in rats deficient in vitamin B_{12} and doubly deficient in vitamin B_{12} and folic acid has been compared with that in vitamin B_{12}-treated controls. The deficient animals have been found to incorporate less isotope into the methyl group of choline than the controls. The animals deficient in folic acid as well as vitamin B_{12} showed less incorporation than animals deficient in vitamin B_{12} alone.

The authors wish to express their thanks to Dr. V. du Vigneaud and Dr. J. R. Rachele for their interest and advice. They also wish to thank Miss Sachi Fujii and Mr. Joseph Albert for the microanalyses.

BIBLIOGRAPHY

1. Welch, A. D., and Nichol, C. A., *Ann. Rev. Biochem.*, **21,** 655 (1952).
2. Bessey, O. A., Lowe, H. J., and Salomon, L. L., *Ann. Rev. Biochem.*, **22,** 553 (1953).
3. Jukes, T. H., and Williams, W. L., in Sebrell, W. H., Jr., and Harris, R. S., The vitamins, New York, **1,** 425 (1954).
4. du Vigneaud, V., and Verly, W. G., *J. Am. Chem. Soc.*, **72,** 1049 (1950).
5. Arnstein, H. R. V., *Biochem. J.*, **47,** p. xviii (1950).
6. Arnstein, H. R. V., *Biochem. J.*, **48,** 27 (1951).
7. du Vigneaud, V., Verly, W. G. L., Wilson, J. E., Rachele, J. R., Ressler, C., and Kinney, J. M., *J. Am. Chem. Soc.*, **73,** 2782 (1951).
8. Verly, W. G., Wilson, J. E., Kinney, J. M., and Rachele, J. R., *Federation Proc.*, **10,** 264 (1951).
9. Verly, W. G., Kinney, J. M., and du Vigneaud, V., *J. Biol. Chem.*, **196,** 19 (1952).
10. Stekol, J. A., Weiss, K. W., and Weiss, S., *Federation Proc.*, **10,** 252 (1951).
11. Arnstein, H. R. V., and Neuberger, A., *Biochem. J.*, **55,** 259 (1953).
12. Arnstein, H. R. V., and Neuberger, A., *Biochem. J.*, **48,** p. ii (1951).
13. Stekol, J. A., Weiss, S., and Weiss, K. W., *Arch. Biochem. and Biophys.*, **36,** 5 (1952).
14. Arnstein, H. R. V., and Neuberger, A., *Biochem. J.*, **50,** p. xxxviii (1952).
15. Stekol, J. A., Anderson, E. I., Weiss, S., and Hsu, P. T., Abstracts, American Chemical Society, 124th meeting, Chicago, 1C (1953).
16. Sakami, W., and Welch, A. D., *J. Biol. Chem.*, **187,** 379 (1950).
17. du Vigneaud, V., Verly, W. G., and Wilson, J. E., *J. Am. Chem. Soc.*, **72,** 2819 (1950).
18. du Vigneaud, V., Ressler, C., Rachele, J. R., Reyniers, J. A., and Luckey, T. D., *J. Nutr.*, **45,** 361 (1951).
19. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136,** 509 (1940).

UREIDOSUCCINIC ACID AS A PRECURSOR OF NUCLEIC ACID PYRIMIDINES IN NORMAL AND TUMOR-BEARING MICE*

By E. P. ANDERSON,† C. Y. YEN, H. GEORGE MANDEL, AND PAUL K. SMITH

(From the Department of Pharmacology, School of Medicine, George Washington University, Washington, D. C.)

(Received for publication, July 6, 1954)

Orotic acid has been established as a precursor of nucleic acid pyrimidines in various organisms (1–6). Wright and coworkers (5) found that ureidosuccinic acid appeared to be a precursor of orotic acid in *Lactobacillus bulgaricus* 09, and more recent work with rat tissue slices (7, 8) and homogenates (9) and with bacterial enzymes (10, 11) has further implicated ureidosuccinic acid in this system. It seems likely, then, that ureidosuccinic acid may serve as a precursor of nucleic acid pyrimidines by way of orotic acid, and it was therefore of interest to investigate the behavior of ureidosuccinic acid *in vivo*, with particular reference to its incorporation into nucleic acids. The present work is a study of the distribution and excretion of labeled ureidosuccinic acid in normal and tumor-bearing mice and of its uptake into the individual nucleic acid pyrimidines of liver and tumor tissue.

EXPERIMENTAL

Ureidosuccinic acid was synthesized, labeled with C^{14} in the ureido carbon, according to the method of Nyc and Mitchell (12).[1] Urea-C^{14} was treated with potassium carbonate to give labeled potassium cyanate, and this was allowed to react with DL-aspartic acid to give C^{14}-ureidosuccinic acid. The over-all yield was 50 per cent based on urea. Increasing the time of interaction of potassium cyanate with aspartic acid to 48 hours and acidifying at a lower temperature made the yield more reproducible. The melting point of the compound was 180–181°, in agreement with the litera-

* A preliminary report of this work was presented at the Nineteenth International Physiological Congress, Montreal, 1953. This investigation was supported by research grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service (No. C-308), from the Damon Runyon Memorial Fund for Cancer Research, Inc. (No. DRIR-42C), and by a contract with the United States Atomic Energy Commission (No. AT(30-1)-1107).

† Present address, National Institutes of Health, Bethesda, Maryland.

[1] The authors are indebted to Curtis L. Brown for technical assistance in the synthetic work.

ture value (12). The purity was checked by paper chromatography in several solvent systems. Freedom from contamination with aspartic acid was demonstrated by the absence of a color reaction with ninhydrin on these chromatograms, and in each case the chromatogram showed a single peak of radioactivity. The specific activity of the C^{14}-ureidosuccinic acid was 0.12 μc. per mg.

Adult CAF_1 mice were employed as experimental animals. The tumor used was sarcoma 37, which had been transplanted into the hind leg. This tumor grows rapidly, reaching maximal size about 7 days after transplantation.

For a preliminary study of the excretion and distribution, groups of three mice were injected intraperitoneally with a neutral aqueous solution of C^{14}-ureidosuccinic acid, each mouse receiving a dose of 30 mg. per kilo. The mice were then placed in a glass metabolism chamber, and urine, feces, and respiratory carbon dioxide were collected for given intervals of time. At the end of the period the animals were killed, and livers, tumors, and pooled viscera were removed. Viscera comprised all internal organs of the body cavity except liver and bladder; the gastrointestinal tract was washed free of its contents before being included. The tissues of both normal and tumor-bearing animals were examined after 2 hour and 24 hour intervals, and those of an additional group of tumor-bearing mice after an 8 hour period.

Excretion Pattern—The urine was assayed for radioactivity on glass plates. The self-absorption correction was found to be negligible, based on urine collected from a group of three saline-injected, tumor-bearing mice, plated along with known amounts of radioactivity. Respiratory carbon dioxide was assayed as barium carbonate, which was deposited on aluminum planchets. A measure of the activity in the collected feces was obtained by direct counts on known weights of the solid material. All counts were made with a methane flow proportional counter.

The activity found in these materials was compared with that of the injected solution, based in each case on similar counting procedures. As a control for the assay on respiratory carbon dioxide, aliquots of the injected solution were converted to carbon dioxide by the wet combustion method described by Weisburger *et al.* (13), and counted on aluminum planchets as barium carbonate.

From the results it could be seen that in both normal and tumor-bearing animals about half of the injected radioactivity was excreted in the urine within the first 2 hours, with not much further excretion for longer intervals of time up to 24 hours. The radioassay of feces showed negligible excretion of the compound by way of the gastrointestinal tract during 24 hours. Fractionation of the urine by paper chromatography in two sol-

vent systems showed some activity in the spot corresponding to ureidosuccinic acid and no detectable activity in that corresponding to urea. However, most of the activity was present in another spot not identical with either of these. This component had the same R_F value in both solvent systems as the product obtained by acid treatment of ureidosuccinic acid, identified by Nyc and Mitchell (12) as 5-(acetic acid)-hydantoin.

The activity in the respiratory carbon dioxide totaled less than 5 per cent of the injected dose for the first 2 hours after isotope administration and increased to about one-fifth of the injected radioactivity by 24 hours. However, the actual level of activity on the barium carbonate plates was so low that the figures are subject to considerable error.

TABLE I

Distribution of Radioactivity at Various Time Intervals after Administration of C^{14}-Ureidosuccinic Acid to Mice

TCA extract	Tissue	2 hrs.		8 hrs.	24 hrs.	
		Normal	Tumor-bearing	Tumor-bearing	Normal	Tumor-bearing
Cold	Liver	488	1904	785	40	175
	Viscera	120	101	55	23	29
	Tumor		0	0		0
Hot	Liver	131	462	655	76	361
	Viscera	37	41	48	52	54
	Tumor		20	32		24

The values are counts per minute per gm. of wet weight of tissue.

Survey of Distribution in Vivo—The tissues from each of these same groups of mice were homogenized and extracted with ice-cold 5 per cent trichloroacetic acid (TCA) to obtain an acid-soluble fraction, and with hot TCA, according to Schneider (14), to gain an estimate of the distribution in the nucleic acid fraction. Aliquots of each tissue extract were plated directly in plastic cups for counting. Self-absorption corrections were applied, determined in each case with TCA extracts of identical tissues from the saline-injected, tumor-bearing controls, plated along with known amounts of radioactivity. The tissue residues remaining after TCA extraction were assayed for radioactivity by direct counts on the solid material.

Table I shows the distribution of activity in the TCA extracts in terms of the counts per minute per gm. of wet weight of tissue. The level of radioactivity in the cold TCA or acid-soluble fraction was highest in the liver, especially at early stages after isotope administration, and also appreciable in the viscera, but no activity could be detected in this frac-

tion of the tumor. In the hot TCA fractions containing the nucleic acids, the level was highest in the liver and considerably lower but nevertheless significant in the tumor. There was also some incorporation into this fraction in the viscera. On an absolute basis, the most active fraction contained a total of about 8 per cent of the injected radioactivity. The other fractions represented 2 per cent or less of the total injected dose.

The analysis of the protein residues remaining after TCA extraction showed negligible activity in all cases.

TABLE II

Incorporation of C^{14}-Ureidosuccinic Acid into Nucleic Acid Pyrimidines in Mice

Experiment No.		Liver			Tumor		
		PNA		DNA	PNA		DNA
		Cytoplasm	Nuclei		Cytoplasm	Nuclei	
1. Tumor-bearing	Uracil	405	277		19	12	
	Cytosine	288	273	9	19	19	16
	Thymine			4			16
2. Tumor-bearing	Uracil	491	225		18	11	
	Cytosine	314	297	13	16	16	16
	Thymine			4			11
3. Normal	Uracil	198	168				
	Cytosine	92	216	3			
	Thymine			1			
4. Normal	Uracil	194					
	Cytosine	109		4			
	Thymine			1			

The values are expressed in counts per minute per micromole of pyrimidine base.

Incorporation into Nucleic Acid Pyrimidines—A more detailed study of this incorporation of ureidosuccinic acid was made in four experiments with larger groups of animals, two groups of twenty-seven and 58 normal mice, and two groups of twenty and 58 mice bearing sarcoma 37.

These animals were given five equal injections of ureidosuccinic acid at approximately 12 hour intervals. The total dosage was 150 mg. per kilo. In the case of the tumor-bearing mice, injections were begun on the 4th day after tumor transplant.

The animals were killed 24 hours after the last injection. Livers and tumors were removed, homogenized, and fractionated into nuclei and cytoplasm in citric acid, as described in previous work (15). In one experiment with normal mice, the livers were not separated into nuclei and

cytoplasm (Experiment 4, Table II). In this case the tissue was frozen in dry ice and alcohol immediately after removal, and homogenized for a longer period; it was then disintegrated by vibration and extracted with alcohol-ether in the same way as the other tissue samples.

The dried tissues were treated according to Hammarsten (16) to extract mixed polynucleotides. These were hydrolyzed in alkali and separated into pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) with lanthanum (16). The solutions of PNA nucleotides were treated with 1 N HCl at 100° for 1 hour to hydrolyze purine nucleotides to free bases. Individual pyrimidine nucleotides and purine bases were then isolated by Dowex 50 ion exchange chromatography by elution with 0.1 N acetic acid and 4 N hydrochloric acid, according to Cohn (17). The pyrimidine nucleotides were further degraded to free bases by hydrolysis in formic acid (18) and purified by starch chromatography (2). The lanthanum-DNA was decomposed with carbonate and the DNA precipitated with acid alcohol as described in previous work (2). This was hydrolyzed with dry HCl in methanol (18), and the pyrimidines further hydrolyzed in formic acid and again purified on starch.

The purity of the isolated pyrimidine bases was checked by ultraviolet absorption characteristics and by the value for $E_{max.}$ per microgram of N per ml. Radioactivity was measured at infinite thinness on glass. Concentrations were determined from ultraviolet extinction coefficients and the specific activities calculated.

Whereas no activity could be detected in the purine fractions, all the nucleic acid pyrimidines were found to be radioactive, as shown in Table II. The most striking differences observed were in the PNA pyrimidines. The liver PNA of tumor-bearing mice showed appreciably more incorporation than did that of normal animals, and was markedly higher than tumor PNA. This confirms the results found in the general survey of TCA nucleic acid fractions and would indicate that the activity in the TCA fractions reflects primarily incorporation into the PNA.

It is interesting to note that the great difference observed between liver and tumor in the level of activity in the acid-soluble fraction was also confirmed by radioassay of the citric acid extracts examined after precipitation of the cytoplasm material with alkali. In the tumor this fraction was also relatively low in activity.

Table II also indicates that in both normal and tumor-bearing animals the incorporation into liver PNA was much greater than that into liver DNA. The figures for the liver DNA pyrimidines are, in fact, subject to appreciable error, especially in the normal animals, since the actual counts were very low.

In tumor the turnover of DNA tended to be somewhat higher than that in liver. Since the activity of the tumor PNA was also comparatively low, there was no great difference between the figures for PNA and DNA in tumor.

In all cases the level of activity in nuclear and cytoplasmic PNA was of approximately the same magnitude. As would be expected from the greater quantity of cytoplasmic PNA present, the total activity in liver PNA in the second experiment on normal mice was essentially the same as that of cytoplasm PNA alone.

There were appreciable differences between the incorporation values for the individual pyrimidine bases in the cytoplasmic PNA of liver, that of

TABLE III

Dilution of Isotope during Incorporation into Nucleic Acid Pyrimidines in Mice

Experiment No.		Liver			Tumor		
		PNA		DNA	PNA		DNA
		Cytoplasm	Nuclei		Cytoplasm	Nuclei	
1. Tumor-bearing	Uracil	0.0342	0.0234		0.0016	0.0010	
	Cytosine	0.0243	0.0231	0.0008	0.0016	0.0016	0.0014
	Thymine			0.0003			0.0014
3. Normal	Uracil	0.0139	0.0119				
	Cytosine	0.0065	0.0152	0.0002			
	Thymine			0.0001			

The values are expressed as ratios of molar specific activity of compound isolated to that of precursor used (C^{14}-ureidosuccinic acid).

uracil being higher than that of cytosine. Such differences were not evident in tumor cytoplasm PNA.

In Table III these findings are expressed as the ratio of molar activity of the compound isolated to that of the precursor, to show the dilution of the isotope. On a molar basis the level of activity in liver PNA is on the order of 0.01 of that of the injected compound in normal mice and 0.02 in tumor-bearing mice. This is a significant incorporation, similar in extent to that of 4-amino-5-imidazolecarboxamide into nucleic acid purines under similar conditions of administration (19), and would indicate ureidosuccinic acid to be a relatively good precursor of liver PNA in the mouse. However, in contrast to the results with the purine precursor, which showed a slightly higher incorporation into tumor PNA, the level of activity in tumor PNA in these experiments is only one-twentieth as great as that in liver PNA of tumor-bearing mice. This emphasizes again the difference between liver and tumor found with this precursor.

DISCUSSION

The pattern of excretion and distribution of ureidosuccinic acid observed in these experiments seems to follow that found by Hurlbert and Potter (3) for orotic acid in tumor-bearing rats. The incorporation picture also agrees with our present knowledge on orotic acid as a nucleic acid precursor and is in agreement with a conversion of ureidosuccinic acid to orotic acid and incorporation by this pathway.

The very low uptake by DNA, for example, and the higher incorporation into PNA are in agreement with findings on orotic acid (2, 15). The excretion of appreciable radioactivity in the respiratory carbon dioxide raises a question as to whether the incorporation of ureidosuccinic acid into DNA could be accounted for merely by contribution of the carbon dioxide pool rather than of the intact molecule. However, in these experiments no activity could be detected in the nucleic acid purines, which would also be expected to draw upon this carbon dioxide pool (20, 21).

The greater turnover of DNA in tumor confirms previous work with various nucleic acid precursors, and may be expected from the higher mitosis rate for tumor tissue.

A higher nucleic acid turnover in non-tumor tissues of tumor-bearing animals, compared with normals, has also been observed by Payne and coworkers (22, 23), using P^{32}, glycine, and formate, by Tyner and coworkers (24) with glycine, and by Conzelman *et al.* (25) with 4-amino-5-imidazolecarboxamide. The differences reported by these workers were found to be primarily due to an increased uptake by DNA, although Conzelman and coworkers (25) also found a small increase in PNA turnover. The results in these experiments indicate an increased incorporation of ureidosuccinic acid by both PNA and DNA in liver of mice bearing sarcoma 37.

The similarity in the incorporation figures for nuclear and cytoplasmic PNA could be expected on the basis of the long isotope administration time in these experiments. The great difference between these cell parts in incorporation of orotic acid, apparent at early stages after isotope exposure (3, 15), has been shown by Hurlbert and Potter to disappear at later stages.

The marked difference between liver and tumor in the level of incorporation of ureidosuccinic acid into PNA is parallel to that observed with orotic acid in rats bearing Flexner-Jobling carcinoma (3); the incorporation into the PNA of this tumor was also low compared to liver, and more activity was found in the tumor nucleic acids than in the acid-soluble fraction of the tumor. In this work (3) the authors postulated that the incorporation in tumor might not proceed from orotic acid itself but rather from products of orotic acid metabolism formed in other tissues. Based on an indirect comparison with results on glycine incorporation, a high uptake and utilization of orotic acid by liver, rather than a low uptake by tumor,

were judged to be the major factors affecting the ratio between these two tissues. It may be that the liver has such a preferential uptake of these precursors that they do not reach the tumor in any great quantity. Studies now in progress on the uptake of ureidosuccinic acid by spleen indicate that this tissue is intermediate between liver and tumor, but more like tumor, in extent of incorporation.

In this connection, it is also of interest that dissimilarities of incorporation were observed between uracil and cytosine in liver cytoplasm PNA but not in tumor cytoplasm PNA. Comparable results have also been found with orotic acid (3, 4), and have been considered to be further evidence for a difference in pathway in the two tissues (3). However, all such differences in level of incorporation and in the relative uptake by the two pyrimidines are influenced by variations in dilutions of the intermediates. Investigations now under way on the acid-soluble precursors should give further information on the relative pool sizes and the rates of the biosynthetic conversions.

Another possible factor is that the tumor cell *in vivo* may actually be relatively impermeable to these precursors in their injected form, and may therefore be dependent upon some other path of incorporation into nucleic acids. The studies *in vitro* by Weed (4) do not show this marked difference between liver and tumor; tumor slices (including several kinds of sarcoma but not sarcoma 37) incorporated orotic acid into nucleic acids more rapidly than did liver slices, and the difference between uracil and cytosine was even greater in tumor than in liver. A difference in intracellular enzyme distribution or action is therefore unlikely, but further studies on the intermediates should also yield information on this point.

SUMMARY

1. Ureidosuccinic acid labeled with C^{14} in the ureido carbon was synthesized from urea-C^{14} in a 50 per cent yield based on urea.

2. Excretion studies with CAF_1 mice showed very rapid urinary elimination of about half the injected radioactivity. Appreciable activity was also found in the expired CO_2.

3. A survey of tissue distribution and a detailed investigation of incorporation into nucleic acids were carried out with normal mice and with mice bearing sarcoma 37. Ureidosuccinic acid was incorporated into all the nucleic acid pyrimidines. A much higher incorporation into liver PNA than into tumor PNA was found, and the activity in the acid-soluble fraction of liver was much above that of tumor.

4. The incorporation into uracil was greater than into cytosine in liver cytoplasm PNA but not in tumor cytoplasm PNA. There were no significant differences in uptake between individual pyrimidines in nuclear PNA or in DNA.

5. The uptake into PNA was very much higher than into DNA in liver. The turnover of tumor DNA was somewhat higher than that of liver DNA.

6. The turnover of both PNA and DNA was higher in liver of tumor-bearing mice than in that of normal animals.

7. There were no great differences between cytoplasm and nuclei in PNA turnover.

8. The pattern of excretion, distribution, and incorporation follows that for orotic acid.

BIBLIOGRAPHY

1. Arvidson, H., Eliasson, N. A., Hammarsten, E., Reichard, P., von Ubisch, H., and Bergström, S., *J. Biol. Chem.*, **179**, 169 (1949).
2. Reichard, P., *Acta chem. Scand.*, **3**, 422 (1949).
3. Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **195**, 257 (1952).
4. Weed, L. L., *Cancer Res.*, **11**, 470 (1951).
5. Wright, L. D., Miller, C. S., Skeggs, H. R., Huff, J. W., Weed, L. L., and Wilson, D. W., *J. Am. Chem. Soc.*, **73**, 1898 (1951).
6. Edmonds, M., Delluva, A. M., and Wilson, D. W., *J. Biol. Chem.*, **197**, 251 (1952).
7. Weed, L. L., and Wilson, D. W., *J. Biol. Chem.*, **207**, 439 (1954).
8. Reichard, P., and Lagerkvist, U., *Acta chem. Scand.*, **7**, 1207 (1953).
9. Cooper, C., and Wilson, D. W., *Federation Proc.*, **13**, 194 (1954).
10. Lieberman, I., and Kornberg, A., *Biochim. et biophys. acta*, **12**, 223 (1953).
11. Lieberman, I., and Kornberg, A., *J. Biol. Chem.*, **207**, 911 (1954).
12. Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, **69**, 1382 (1947).
13. Weisburger, J. H., Weisburger, E. K., and Morris, H. P., *J. Am. Chem. Soc.*, **74**, 2399 (1952).
14. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
15. Anderson, E. P., and Åqvist, S. E. G., *J. Biol. Chem.*, **202**, 513 (1953).
16. Hammarsten, E., *Acta med. Scand.*, suppl. 196, 634 (1947).
17. Cohn, W. E., *Science*, **109**, 377 (1949).
18. Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **176**, 715 (1948).
19. Carló, P.-E., and Mandel, H. G., *J. Biol. Chem.*, **201**, 343 (1953).
20. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.*, **173**, 69 (1948).
21. Buchanan, J. M., Sonne, J. C., and Delluva, A. M., *J. Biol. Chem.*, **173**, 81 (1948).
22. Payne, A. H., Kelly, L. S., and White, M. R., *Cancer Res.*, **12**, 65 (1952).
23. Payne, A. H., Kelly, L. S., Beach, G., and Jones, H. B., *Cancer Res.*, **12**, 426 (1952).
24. Tyner, E. P., Heidelberger, C., and LePage, G. A., *Cancer Res.*, **13**, 186 (1953).
25. Conzelman, G. M., Jr., Mandel, H. G., and Smith, P. K., *Cancer Res.*, **14**, 100 (1954).

(*From*

The
some
whole
parts.
nervou
as wel
may d
differe
as of l

Thi
brain
GDH)
0.2 to

Qua
made
These
dissect
been r
mon's
and ce
(1) an
tracte

Enz
on the
at 340
techni
tomed
snugly

* Su
Commi
this wo

XUM

THE QUANTITATIVE HISTOCHEMISTRY OF BRAIN

IV. LACTIC, MALIC, AND GLUTAMIC DEHYDROGENASES*

By JACK L. STROMINGER and OLIVER H. LOWRY

(From the Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri)

(Received for publication, September 13, 1954)

The brain is an exceedingly complex histological structure, and, while some information is available concerning the metabolism of brain as a whole, very little is known concerning the metabolism of its component parts. In view of morphological and functional differences within the nervous system, it seems likely that the axons, dendrites, and cell bodies, as well as glial elements and even homologous parts of different neurons, may differ in their metabolic capacity. Previous reports indicate marked differences in the quantitative distribution of a number of enzymes, as well as of lipide and phosphorus fractions (1–6).

This is a report of the histochemical distribution in a few regions of the brain of lactic, malic, and glutamic dehydrogenases (LDH, MDH, and GDH). For this purpose sensitive and simple micromethods (requiring 0.2 to 3 γ of dried brain) for assaying these enzymes were developed.

Materials and Methods

Quantitative measurements of the activity of each enzyme have been made on relatively minute pieces of young adult rabbit brain or cord. These pieces were cut out from frozen-dried histological sections under a dissecting microscope to provide histologically distinct samples which had been neither fixed nor embedded (1). Samples were obtained from Ammon's horn, cerebellar vermis, striate cortex, hypothalamus, retina, medulla, and cervical spinal cord. The dissected fragments (0.2 to 5 γ) were weighed (1) and transferred directly to assay tubes. Some samples were also extracted with alcohol and hexane to obtain the fat-free weights (1).

Enzyme Measurements—The enzyme methods that were used are based on the rate of reduction of diphosphopyridine nucleotide (DPN^+) measured at 340 mμ (7) in a Beckman spectrophotometer. Special microchemical techniques and tools have been described previously (2). The flat bottomed assay tubes were made from 7 mm. Pyrex tubing selected to fit snugly in a special black plastic holder (2). Selection for uniform inner

* Supported in part by a grant from the American Cancer Society through the Committee on Growth of the National Research Council. A preliminary account of this work has previously been presented (*Federation Proc.*, **11**, 295 (1952)).

XUM

diameter (0.474 cm. ± 1 per cent) was based on readings with a colored solution.

The tube containing the tissue fragment is placed in ice water and the complete substrate reagent (70 to 250 μl.) is added. After 1 minute the tube is tapped or gently vibrated (2) and placed in a rack in a water bath at 32° ± 0.2°. Each tube is wiped and read after 1 minute for temperature equilibration and again after appropriate intervals. With a light path of 0.474 cm. and 100 μl. of substrate a change in optical density of 0.2 per hour corresponds to $(0.2 \times 10^{-4})/(6280 \times 0.474) = 6.73 \times 10^{-9}$ mole of DPN^+ reduced per hour ($\epsilon = 6280$ (8)).

The substrate reagents are prepared in quantity without the DPN^+ and are stored frozen. The DPN^+ is added just before use as a dry powder as follows: 0.7, 1.3, and 3.5 mg. per ml. (calculated as pure DPN^+) for LDH, MDH, and GDH, respectively. In this study the substrates consisted of (*a*) 85 per cent racemic sodium lactate (Mallinckrodt Chemical Works, St. Louis), (*b*) potassium L-malate prepared from L-malic acid (Nutritional Biochemicals Corporation, Cleveland), which had been recrystallized (from warm ethyl acetate by the addition of petroleum ether) to remove an inhibitor present in some samples, and (*c*) monosodium L-glutamate (Nutritional Biochemicals Corporation). The DPN^+ was either 65 or 90 per cent (Sigma Chemical Company, St. Louis).

The composition of the buffer-substrate mixture for each enzyme measurement is given in Table I, with the amounts of tissue convenient to use in each assay. At the high dilutions of rabbit brain employed neither reduction of DPN^+ by endogenous substrates nor reoxidation of reduced DPN^+ was observed. Keto acid formation has been shown to equal exactly DPN^+ reduction under the assay conditions.[1]

The recommended assay conditions are based on studies of the properties of rabbit brain LDH, MDH, and GDH (Table I, Fig. 1). All of these enzymes are measured in the less favorable direction as far as equilibrium is concerned. However, in the case of LDH and MDH, both the high substrate levels and the high pH result in initial velocities of the same order of magnitude as those in the reverse direction.[2]

[1] Robins, E., Roberts, N. R., Eydt, K. M., and Lowry, O. H., to be published.

[2] The maximal initial velocities depend on both the equilibrium constants and the respective K_m values (10). A high pH optimum and differences in K_m have a considerable effect on the ratio of maximal initial velocities. For example, in the case of LDH, at pH 7, with equal DPN^+ and reduced DPN^+, the equilibrium ratio of lactate to pyruvate is about 30,000:1 (11). However, at the pH optimum of 9.2 this equilibrium ratio is much more favorable, about 200:1. In addition, the K_m for lactate is about 500 times larger than the K_m for pyruvate. As a result, at pH 9.2, the initial velocities should be approximately equal. A similar result is obtained for MDH with a recent value for the MDH equilibrium constant (12).

TABLE I

Constants and Assay Conditions for Three Dehydrogenases of Rabbit Brain

	LDH	MDH	GDH
Constants of enzymes			
K_m for substrate, m_M	5.0*	1.3	0.85
" " DPN$^+$, m_M	0.056	0.3	1
Optimal pH	9.2	10.2	8.1
Q_{10}	2.2	2.3	
Assay conditions			
Buffer (at pH optimum of enzymes), M	0.1 glycine	0.1 glycine	0.05 Veronal
Substrate concentration, M	0.2 (D, L)	0.2 (L)	0.03 (L)
DPN$^+$ concentration, m_M	1	2	5
Activity of whole rabbit brain, *moles per kilo dry weight per hr.*†	7	32	0.8
Weight of dry brain for convenient rate in 100 μl., γ	1.5	0.3	10

* Calculated for concentration of L-lactate.

† Rabbit brain contains 22 per cent dry weight.

Under the conditions chosen all three reactions are linear until the optical density increase exceeds 0.200. The assays are proportional to time up to 2 hours and to the amount of rabbit brain homogenate added, provided that the optical density increase does not exceed 0.200 (Fig. 2). The sensitivity of the methods is indicated by the high dilutions employed.

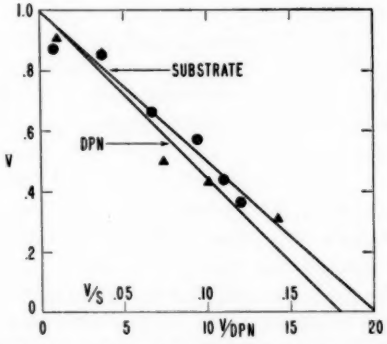


FIG. 1. Substrate-velocity and DPN-velocity relationships for LDH. The plot is made according to Eadie (9). V is expressed as a fraction of $V_{max.}$. S and DPN$^+$ are expressed in millimoles per liter. The intercepts on the abscissa are the reciprocals of the respective K_m values in the same terms.

The sensitivity could be increased by reducing the volume and by increasing the incubation temperature to 38° and the incubation time. In the case of MDH, such modifications should permit analysis of 0.0015 γ of brain (dry weight); this is the weight of a single moderate sized cell. GDH is much less active than the other two enzymes in brain. Nevertheless, by using an incubation time of 1 hour and a 70 μl. volume about 3 γ of brain (dry weight) are conveniently analyzed.

Carbonyl-trapping reagents such as cyanide, hydrazine, and hydroxylamine have been found by previous workers employing other assays to accelerate reaction rates of these enzymes by removing the keto acids formed. Under the conditions proposed, however, all of these substances

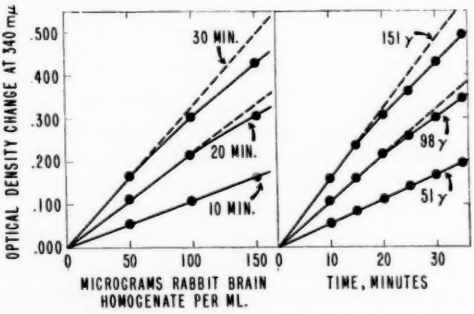


FIG. 2. Linearity of assay for LDH activity with respect to quantity of rabbit brain and time. These assays were carried out in a 1 ml. volume. The concentration of wet brain during assay is indicated.

were inhibitory. In 0.5 M cyanide (at pH 9.2) which has been used previously in an assay for lactic dehydrogenase in which oxygen consumption was measured (13), no lactic dehydrogenase activity could be demonstrated spectrophotometrically. With 1.1, 2.5, and 4.5 mmoles per ml. of cyanide the velocity was 43, 21, and 8 per cent, respectively, of the rate without cyanide. (A small increment in optical density owing to complex formation between DPN^+ and cyanide (14) was observed. Lactate and brain homogenate were not added until the optical density became constant.) Nicotinamide had no effect on the reaction rates.

Purification of Enzymes and Addition of Purified Enzymes to Assay System with Homogenate—The estimation of enzyme concentration based on measurement of a catalytic property of the enzyme would be subject to serious errors if accelerators or inhibitors of reaction rate were present in the tissue being analyzed. The three enzymes from rabbit brain homogenate have, therefore, been partially purified, and assays have been carried out to see

whether the activities of the purified enzymes and homogenates are strictly additive when combined.

LDH and MDH were purified together, each 10- to 15-fold (based on protein), by reprecipitation with $(NH_4)_2SO_4$ between 2.1 and 2.9 M at pH 7.5. GDH precipitated between 1.25 and 1.70 M. Several fractionations between these limits yielded a preparation 100-fold purified in 90 per cent yield. For all three enzymes the sum of the rates obtained for purified enzyme alone or for homogenate alone was equal (within analytical error) to the rate obtained when the assay was performed with purified enzyme plus homogenate.

Effects of Freezing, Drying, and Storage on Apparent Enzyme Concentration—MDH and LDH in homogenates showed no loss of activity on freezing and drying. In fact, in some homogenate samples activity increased 5 or 10 per cent on freezing and another 5 or 10 per cent on freeze-drying. Since the initial values were low in these samples, these increases can probably be attributed to incomplete homogenization and better disruption of the tissue by freeze-drying.

These two enzymes also survived storage quite well in the frozen-dried state. Loss of activity on standing at room temperature (25°) for 2 days was 6 to 8 per cent both in homogenates and in samples from sections, and homogenates lost about 40 per cent during 2 weeks at room temperature. No significant decrease was observed on storage at −20° for 13 days. Samples of zona radiata of Ammon's horn which had been stored at −20° for about a year had 92 per cent of the LDH and 90 per cent of the MDH activity of newly prepared samples (from another rabbit).

The stability of GDH was not tested so extensively. However, 1 year-old samples of sections from radiata of Ammon's horn contained 125 per cent of the activity of a 1 week-old sample from another rabbit.

The analyses reported in this paper were carried out on samples from sections stored in the deep freeze for a maximum of 2 weeks and exposed to room temperature for a maximum of 8 hours.

Results

Ammon's Horn—This is a part of the hippocampus (allocortex) which is divided sharply into six layers of the following thickness and composition: (1) alveus, 200 μ, myelinated fibers, (2) oriens, 250 μ, non-myelinated axons and dendrites, (3) pyramidalis, 50 μ, densely packed cell bodies of small pyramidal cells, (4) radiata, 400 μ, closely packed dendrites, (5) lacunosum, 150 μ, dendrites plus myelinated fibers, (6) molecularis, 200 μ, neuropile, *i.e.* terminal arborizations of dendrites and axons.

The dendrite and non-myelinated fiber areas are richest in both LDH and MDH (Table II), especially on a fat-free basis (Fig. 3). It is to be noted

TABLE II

Dehydrogenases of Ammon's Horn

The enzyme activities are expressed as moles per kilo dry weight per hour. The values are the averages of five to eight analyses each (standard errors indicated). Fat-free weight is per cent of total dry weight.

	LDH	MDH	GDH	Fat-free weight	MDH / LDH	LDH / GDH
Alveus (myelinated fibers)	6.94 ±0.37	24.8 ±2.2	0.662 ±0.055	38	3.6	10
Oriens (axons and dendrites)	18.2 ±0.8	63.0 ±3.2	0.638 ±0.061	59	3.5	28
Pyramidalis (cell bodies)	21.8 ±0.6	54.8 ±1.9	0.363 ±0.016	78	2.5	60
Radiata (dendrites)	19.8 ±0.7	57.1 ±0.9	0.477 ±0.055	62	2.9	41
Lacunosum (dendrites and myelinated fibers)	14.5 ±0.4	50.4 ±1.8	0.443 ±0.053	49	3.5	33
Molecularis (neuropile)	15.3 ±0.4	61.0 ±1.2	0.962 ±0.080	61	4.0	16

that on a fat-free basis the cell body layer is not conspicuously rich in either enzyme. In fact the myelinated fiber layer, which is quite low in both activities on a total dry weight basis, is almost as rich on a fat-free basis as the cell body layer (Fig. 3). The distribution of these two enzymes in Ammon's horn resembles that previously reported for aldolase, acid phosphatase, and adenosinetriphosphatase (4).

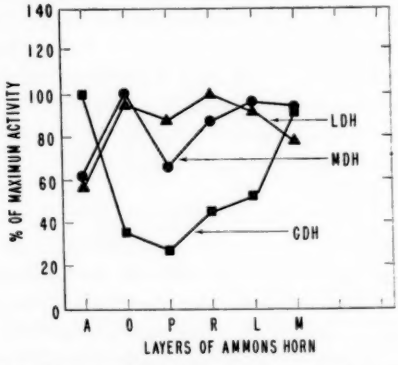


FIG. 3. Distribution of enzymes in layers of Ammon's horn, expressed in terms of activity per fat-free dry weight and plotted in per cent of maximal activity for each enzyme. *A* = alveus, *O* = oriens, *P* = pyramidalis, *R* = radiata, *L* = lacunosum, *M* = molecularis (see the text for description of the layers).

GDH has an entirely different distribution. The myelinated fibers and neuropile are relatively very rich in GDH. The cell body layer, the lowest area, had only one-fourth the activity of the myelinated fibers on a fat-free basis (Fig. 3).

Cerebellum, Striate Cortex, Supraoptic Nucleus, and Hypothalamus (Table III)—The cell body (granular) and molecular layers of cerebellum and striate cortex are very similar to corresponding areas in Ammon's horn in regard to the activities of LDH and MDH. The supraoptic nucleus and the hypothalamic area immediately adjacent to it were analytically almost indistinguishable and contain lower activities of both LDH and MDH than the other cell body and non-myelinated fiber-dendrite areas. The relative proportions of activities are also slightly different, the MDH:LDH ratios being 4.4 and 4.7, compared to values of 2.5 to 4.0 in Ammon's horn, cerebellum, and striate cortex. Abood *et al.* have reported (15) that respiratory and glycolytic activity in supraoptic nucleus is the same as that found in other hypothalamic areas.

Optic Nerve and Retina—Optic nerve and optic tract (obtained from the hypothalamic sections) contain unusually high LDH activity (Table III). These fiber tracts are as rich in this enzyme on a dry weight basis as cell body and molecular layers elsewhere and contain about twice as much activity as these areas on a fat-free dry weight basis. The LDH values are 2 to 6 times higher than those of other myelinated fiber tracts analyzed (Table III). Malic dehydrogenase is very low in optic nerve and tract, perhaps one-fourth of the level in alveus. The MDH:LDH ratio of 0.4 is extraordinarily different from all other regions of the nervous system examined. Consequently, some relatively crude data were obtained for the layers of rabbit retina. Only a partial separation of layers was attempted. Nevertheless, most of the data were surprisingly consistent and striking. The retinal fibers (Layer 9) and the adjacent Layers 6, 7, and 8 (composed of the ganglion cells, whose axons form the retinal fiber tract and optic tract, the inner reticular (synaptic) layer, and the inner nuclear layer of bipolar cells) have a composition in keeping with the unusual composition of the optic tract. Layers 6 to 8 have the highest LDH activity found in this study and have an MDH:LDH ratio of about 0.4.

The next two layers, the outer reticular and outer nuclear layer, have a much lower LDH activity and an extremely high MDH activity which gives a ratio of 4.9. The samples of the rod layer appeared to fall into two groups, one with low MDH activity and the other with extraordinarily high amounts of this enzyme and an MDH:LDH ratio of at least 14. From data on monkey retina[3] it is clear that the high and low MDH

[3] Lowry, O. H., and Roberts, N. R., to be published.

activities correspond to the inner and outer rod segments. The chorioid is low in both enzymes.

Myelinated Fibers—The other myelinated fiber tracts analyzed are not similar to optic nerve and tract (Table III). They seem to group two

TABLE III

Dehydrogenases in Various Regions of Rabbit Brain

The values are averages of usually six analyses each and are expressed as moles per kilo dry weight per hour (standard errors indicated).

	LDH	MDH	MDH/LDH		LDH	MDH	MDH/LDH
Cerebellum				Retina			
Molecularis	16.5 ±0.6	60.2 ±4.2	3.7	Fibers	28.6 ±0.6	11.2 ±0.5	0.39
Purkinje cell layer	17.2 ±1.0	60.2 ±4.8	3.5	Ganglion + inner reticular + inner nuclear	51.5 ±3.5	27.2 ±1.1	0.53
Granular layer	14.7 ±0.5	58.5 ±4.2	4.0	Outer reticular + outer nuclear	19.5 ±0.6	95.2 ±5.4	4.9
Medulla (myelinated fibers)	6.94 ±0.24	19.9 ±1.3	2.9	Rods* (*a*)	11.2	157	14
Striate cortex				" (*b*)	4.3	11	2.5
Molecularis (I)	16.0 ±0.3	55.3 ±1.3	3.5	Chorioid	5.33 ±0.21	9.67 ±0.43	1.8
Layer II	15.8 ±0.4	50.7 ±0.7	3.2	Pyramidal tract (medulla)	4.89 ±0.18	13.1 ±0.18	2.7
" V	15.7 ±0.3	51.2 ±1.7	3.3	Spinal cord			
" VIII (myelinated fibers)	4.37 ±0.15	11.0 ±1.0	2.5	Posterior columns	3.87 ±0.12	8.08 ±0.69	2.1
Hypothalamic area				Ventral root	2.59 ±0.14	8.13 ±0.42	3.1
Hypothalamus	9.25 ±0.27	43.7 ±1.7	4.7	Dorsal "	2.66 ±0.28	7.11 ±0.53	2.7
Supraoptic nucleus	10.55 ±0.34	46.8 ±0.9	4.4	" " ganglion	9.81 ±0.34	36.1 ±1.1	3.7
Optic tract	15.8 ±0.22	4.93 ±0.33	0.31				
" nerve	16.4 ±0.4	7.66 ±0.28	0.47				

* The values for the rod layer fell in two groups which have been arbitrarily averaged separately (see the text).

ways: (1) those which are relatively low in both MDH and LDH (Layer VII of striate cortex, ventral and dorsal root, pyramids, posterior column) and (2) those which are relatively high in both MDH and LDH (myelinated layer of cerebellum, alveus of Ammon's horn). There did not seem to be any simple relationship to the content of total lipide. In sections of a dor-

sal root ganglion in which no nerve fibers could be seen the activity of both LDH and MDH was considerably higher than in the dorsal root, but was substantially lower than in other cell body areas.

Aldolase and Fumarase in Myelinated Fibers—Some of the myelinated fibers were analyzed for another glycolytic enzyme, aldolase, and another enzyme of the Krebs cycle, fumarase. In general LDH activity was paralleled by aldolase activity (LDH-aldolase ratio relatively constant) and

TABLE IV

Aldolase, Fumarase, and Relationships with LDH and MDH of Five Fiber Tracts

Data for aldolase and fumarase expressed as moles per kilo fat-free weight per hour. They are the average of about six analyses each (standard errors indicated). Fat-free weight is per cent of total dry weight.

	Fat-free weight	Aldolase* (A)	Fumarase* (F)	$\frac{MDH}{LDH}$	$\frac{F}{A}$	$\frac{LDH}{A}$	$\frac{MDH}{F}$	$\frac{LDH}{A}$ adjusted†
Retinal fiber layer	39.2	12.0 ± 0.3	10.6 ±0.2	0.39	0.88	6.1	2.5	6.5
Optic tract	28.7	7.8 ± 0.1	7.0 ±0.2	0.31	0.90	7.1	2.5	7.4
Cerebellar myelinated fibers	32.3	5.0 ± 0.4	20.4 ±2.7	2.9	4.1	4.3	3.0	6.2
Posterior columns	24.0	3.24 ± 0.12	10.0 ±0.3	2.1	3.1	5.0	3.4	6.7
Alveus of Ammon's horn	38.3	4.3‡	16.6‡	3.6	3.9	4.2	3.9	6.9

* These determinations were kindly carried out on our material by Dr. Mei-Ling Wu using methods previously described (3).

† Calculated by subtracting 2.5 per cent of the malic dehydrogenase values from the aldolase values before calculating the ratios (see the text).

‡ Data from Lowry *et al.* (4).

MDH activity was paralleled by fumarase activity (MDH-fumarase ratio relatively constant) (Table IV).

DISCUSSION

It will be noted that very high activities are obtained for both LDH and MDH with the assay systems employed. The MDH activity calculated at 38° would be 60 moles of DPN^+ reduced (or malate oxidized) per kilo dry weight per hour. This may be compared to earlier values for MDH based on oxygen consumption, *e.g.* 3 moles per kilo dry weight per hour for rabbit brain (16) or mouse brain (17), and to the maximal rate of glycoly-

sis or respiration in intact brain, neither of which is more than 0.5 mole per kilo dry weight per hour. Even at pH 7 both LDH and MDH activity are quite high compared to the over-all metabolism. The earlier measurements of these dehydrogenases were undoubtedly limited by the accessory enzymes required in more complex systems, inability to work at an optimal pH, and some accumulation of keto acids in spite of precautions to remove them. However, a recent determination of GDH in rat brain (18), 0.4 mole per kilo dry weight per hour, also based on oxygen consumption, is in fair agreement with our value.

In regard to the histochemical data, the surprisingly high activity of several enzymes in the myelinated layer of Ammon's horn (4), and the high respiratory and glycolytic activity of white matter in general (19), have been previously noted. This study shows that LDH and MDH may also be rather active in fiber tracts, particularly when calculated on a fat-free basis. The large differences found among different myelinated tracts indicate that different types of fibers may vary widely in their metabolism. Clearly the histological composition of each tract in regard to both fibers and glia will need to be evaluated before enzymes can be safely assigned to particular structures in these tracts.

There is some suggestion from the data presented that metabolic activities in nerve fibers may mirror in part the activities in the cell bodies from which the fibers originate. For example, the low MDH and LDH activity in dorsal root fibers is paralleled by relatively low activity in dorsal root ganglion cell bodies, and the high LDH and MDH activity of optic nerve and tract is reflected in similar findings in the retinal ganglion cell layer. Nevertheless, it also seems probable that fibers have a special metabolism, not related to the metabolism of the cell bodies. The very high GDH activity in the myelinated layer contrasted with very low activity in the cell body layer of Ammon's horn may be such an example.

Warburg, Posener, and Negelein (20) first reported that both glycolysis and respiration in intact retina were unusually high. They stated (1924), "If we consider how different the formed elements of the retina are, then we can hardly assume that all elements contribute in the same measure to the special metabolism of the organ. But if we compute the glycolytic action of the organ on its individual parts—perhaps on the sensory epithelium—then we arrive at values which are incommensurable with the metabolism of other body cells." The present data for LDH and MDH seem to confirm at least the first part of this speculation. High LDH activity was found only in ganglion (and inner nuclear) cells and their axons. Since these cells and axons also have a very low MDH activity, they presumably have a predominantly glycolytic metabolism. Deeper in the retina are structures (outer nuclear cells, rods, pigment epithelium) with

an unusually high MDH activity and a low LDH activity. Presumably their metabolism is predominantly oxidative. It seems probable, therefore, that the high glycolytic and respiratory activities of intact retina are manifestations of metabolic processes occurring in different cells, and the high DPN^+ content of all retinal layers (21)[4] may be related to different dehydrogenases in different cells. This unusual chemical organization and the fact that the retina is a particularly accessible portion of the central nervous system having a highly organized structure suggest that valuable information about the chemical relations of a special multineuron chain might be learned here and invite further quantitative histochemical study.

It would be a great simplification in studies of this kind if it were possible to use the measurement of a single enzyme in a metabolic pathway as a measure of the extent to which that metabolic pathway is functionally active. This not only requires the reasonable but unproved assumption that enzyme activity *in vitro* is directly related to functional activity *in vivo*, but also requires that the enzymes along a given pathway be present in a nearly constant ratio unless there is an intervening fork in the metabolic path. Some support for this idea is provided in the approximate parallelism noted between LDH and aldolase and also between MDH and fumarase in fiber tracts of widely different absolute enzyme activities (Table IV). Since aldolase is on the pathway leading to pyruvate for both oxidation and lactic acid formation, strict parallelism between aldolase and LDH might not be expected. Instead, aldolase activity should be a function of both LDH and of the citric acid cycle enzymes. The data are actually in good agreement with the empirical equation, aldolase = LDH/7 × MDH/40 (Table IV).

SUMMARY

1. Micromethods for determination of LDH, MDH, and GDH in 3 γ of brain (dry weight) or less have been developed. The assays are based on measurement of DPN^+ reduction in the presence of appropriate substrate. Optimal assay conditions were established through kinetic studies of these enzymes in rabbit brain.

2. Quantitative data have been obtained for LDH and MDH in six layers of Ammon's horn, four layers of cerebellum, four layers of striate cortex, supraoptic nucleus, hypothalamus, optic nerve and tract, four layers of retina, four additional myelinated fiber tracts and dorsal root ganglion cells. GDH has been determined only in the layers of Ammon's horn.

3. The distribution of LDH and MDH in Ammon's horn parallels the

[4] The assumption that the DPN^+ content of the retinal fibers is comparable to that of other nerve fibers and therefore low (21) may not be valid, in view of the very high lactic dehydrogenase in these fibers.

XUM

distribution of aldolase. The non-myelinated fiber and dendrite layers are particularly rich in these activities. GDH, in sharp contrast, occurs principally in the myelinated fibers and molecular layer of Ammon's horn.

4. Three types of fiber tracts have been distinguished on the basis of LDH and MDH activity.

5. Retinal ganglion cells and their axons (optic nerve) are very rich in LDH and very poor in MDH. Some structures deeper in the retina have a high MDH activity and a low LDH activity. The high glycolytic and respiratory rates in intact retina are, therefore, probably manifestations of different metabolic processes in different cells.

BIBLIOGRAPHY

1. Lowry, O. H., *J. Histochem. and Cytochem.*, **1,** 420 (1953).
2. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M.-L., and Farr, A. L., *J. Biol. Chem.*, **207,** 1 (1954).
3. Lowry, O. H., Roberts, N. R., Wu, M.-L., Hixon, W. S., and Crawford, E. J., *J. Biol. Chem.*, **207,** 19 (1954).
4. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M.-L., Farr, A. L., and Albers, R. W., *J. Biol. Chem.*, **207,** 39 (1954).
5. Robins, E., Smith, D. E., and McCaman, R. E., *J. Biol. Chem.*, **204,** 927 (1953).
6. Robins, E., and Smith, D. E., *Res. Publ. Assn. Res. Nerv. and Ment. Dis.*, **32,** 305 (1953).
7. Warburg, O., *Ergeb. Enzymforsch.*, **7,** 210 (1938).
8. Ohlmeyer, P. Z., *Biochem. Z.*, **297,** 66 (1938).
9. Eadie, G. S., *J. Biol. Chem.*, **146,** 85 (1942).
10. Kornberg, A., *J. Biol. Chem.*, **182,** 779 (1950).
11. Neilands, J. B., *J. Biol. Chem.*, **199,** 373 (1952).
12. Stern, J. R., Ochoa, S., and Lynen, F., *J. Biol. Chem.*, **198,** 313 (1952).
13. Dewan, J. G., and Green, D. E., *Biochem. J.*, **32,** 626 (1938).
14. Colowick, S. P., Kaplan, N. O., and Ciotti, M. M., *J. Biol. Chem.*, **191,** 447 (1951).
15. Abood, L., Cavanaugh, M., Tschirgi, R. D., and Gerard, R. W., *Federation Proc.*, **10,** 3 (1951).
16. Green, D. E., *Biochem. J.*, **30,** 2095 (1936).
17. Potter, V. R., *J. Biol. Chem.*, **165,** 311 (1946).
18. Copenhaver, J. H., Jr., McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, **183,** 73 (1950).
19. Snyder, J., Abood, L. G., and Gerard, R. W., *Federation Proc.*, **11,** 151 (1952).
20. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, **152,** 309 (1924).
21. Anfinsen, C. B., *J. Biol. Chem.*, **152,** 279 (1944).

QUERCETIN GLYCOSIDES OF GRIMES GOLDEN APPLE SKIN

By H. W. SIEGELMAN

(From the Biological Sciences Branch, Agricultural Marketing Service, United States Department of Agriculture, Beltsville, Maryland)

(Received for publication, September 17, 1954)

Flavonoid compounds are responsible for part of the pigmentation of apple skin. Sando (1, 2) pointed out the relation between the color of apple skin, maturity, market acceptance, keeping quality, and scald and identified quercetin, quercetin 3-galactoside (hyperin), and cyanidin 3-galactoside (idaein) from apple skin. Duncan and Dustman (3) also identified idaein from apple skin.

The present paper describes the separation and identification of the flavonoid complex of Grimes Golden apple skin by paper chromatographic methods. This work is part of an investigation on apple scald, a storage disorder of apples characterized by browning of the skin.

EXPERIMENTAL

Grimes Golden apples were peeled with a small apple-peeling machine. The peel was heated for 3 minutes in a steam bath, and the adhering flesh tissue was removed by scraping with a porcelain spatula. The skin was dried in a draft oven at 65° and ground to pass a No. 40 mesh screen.

100 gm. of the ground skin were extracted with three successive portions of 50 per cent aqueous methanol (5 ml. per gm.) by blending for 5 minutes. The combined extracts were filtered through Hyflo Super-Cel and concentrated under reduced pressure on a steam bath to about 200 ml. This concentrated aqueous solution was then subjected to continuous liquid extraction with ethyl ether for 14 days, which was followed by continuous ethyl acetate extraction for 7 days. The ether and ethyl acetate extracts were each transferred to methanol and are hereinafter referred to as the ethyl ether and ethyl acetate extracts.

Whatman No. 3 paper (18 × 22 inches) was washed (4) with 200 ml. of water per sheet. A total of 5 ml. per sheet of the ethyl ether and ethyl acetate extracts was applied repeatedly in 1 ml. portions each to separate sheets as a 1 cm. streak across the narrow width of the paper. The papers were developed by the descending method with distilled water in a chromatographic cabinet and dried at room temperature. The bands were marked under a long wave ultraviolet lamp, cut out, and eluted with 50 per cent ethanol for 24 hours. The eluates were concentrated with heat on a steam bath. The concentrated eluates were applied to washed sheets of What-

man No. 3 MM as described above, developed with *n*-butanol-acetic acid-water (5), and dried with heat in a fume hood. The resolved bands were marked, eluted, and concentrated as before. The individual bands were further purified by chromatographing again with the *n*-butanol-acetic acid-water solvent, applied as spots on Whatman No. 1 papers, developed with several solvents, and cochromatographed with authentic compounds.

A 1 ml. portion of each concentrated eluate was hydrolyzed with 2 N H_2SO_4 (1 ml.) and methanol (0.1 ml.) by heating at 100° under reflux for 1 hour. The hydrolyzed mixture was cooled in an ice bath for 1 hour and the aglycone portion precipitated from solution. The aglycone portion was separated by centrifugation of the mixture and identified by R_F values in

TABLE I

Preliminary Separation of Ethyl Ether and Ethyl Acetate Extracts of Grimes Golden Apple Skin

In Tables I to IV the following abbreviations for colors are used: B. = brown; G. = green; P. = purple; R. = red; Y. = yellow.

Extract	Band No.	R_F in H_2O (9)*	Color reactions in		
			Visible light	Ultraviolet light	Ultraviolet light + NH_3 vapor
Ethyl ether	1	0.01	Y.	Y.	Y.
" "	2	0.19	"	B.	B.-Y.
" "	3	0.28	"	P.-B.	"
" acetate	4	0.33	"	B.	"

* Bibliographic reference.

various solvents and by color reactions. The liquid portion was neutralized with saturated aqueous barium hydroxide and the barium sulfate precipitate removed by centrifugation. The liquid was concentrated under reduced pressure to about 0.1 ml. for identification of the sugars by paper chromatography.

Ultraviolet absorption spectra of authentic samples were determined in 95 per cent ethanol with the Cary recording spectrophotometer. The ultraviolet absorption spectra of the apple skin components were determined with the Beckman model DU spectrophotometer by the method of Gage and Wender (6), except that 95 per cent ethanol was the eluting solvent.

Results

Preliminary Separation—The ethyl ether extract separated into three prominent yellow bands with the water solvent (Table I). When these

individual bands were chromatographed with the butanol-acetic acid solvent, Bands 1 and 3 remained homogeneous, but Band 2 resolved into three contiguous bands designated as Bands 2.1, 2.2, and 2.3 (Table II).

The ethyl acetate extract yielded only one yellow band with the water solvent (Table I), which remained homogeneous on development with the butanol-acetic acid solvent (Band 4, Table II).

TABLE II

R_F Values and Color Reactions of Quercetin Components of Grimes Golden Apple Skin Extracts and Authentic Quercetin Compounds

Compound	R_F values in				Color reactions in		
	H_2O (9)*	*n*-BuOH-27 per cent aqueous HOAc (1:1) (5)*	*m*-Cresol-HOAc-H_2O (50:2:48) (15)*	PhOH-H_2O (73 per cent aqueous) (5)*	Ultraviolet light	Ultraviolet light + NH_3 vapor	Ultraviolet light + $AlCl_3$ spray (10)*
Skin components							
Band 1	0.01	0.75	0.26	0.33	Y.	Y.	Y.-G.
" 2.1	0.12	0.73	0.40	0.56	B.	B.-Y.	Y.
" 2.2	0.08	0.79	0.42	0.54	"	"	"
" 2.3	0.09	0.83	0.46	0.64	"	"	"
" 3	0.27	0.81	0.42	0.56	P.-B.	"	"
" 4	0.34	0.78	0.13	0.44	B.	"	"
Authentic compounds							
Quercetin	0.01	0.75	0.26	0.34	Y.	Y.	Y.-G.
Quercimeritrin	0.03	0.55	0.23	0.40	"	"	"
Hyperin	0.13	0.73	0.40	0.57	B.	B.-Y.	Y.
Isoquercitrin	0.13	0.75	0.37	0.52	"	"	"
Quercitrin	0.27	0.81	0.43	0.56	P.-B.	"	"
Rutin	0.33	0.79	0.13	0.45	B.	"	"

* Bibliographic reference.

R_F Values and Color Reactions—The six separated components were chromatographed in several carefully chosen solvent systems in comparison with authentic compounds (Table II).

Sugar and Aglycone Portions—The acid hydrolysis products of each band except Band 1 consisted of the aglycone quercetin and one or two sugars. Band 1 contained no sugar (Tables III and IV).

Ultraviolet Absorption Maxima—The ultraviolet absorption maxima for the six bands and authentic samples in 95 per cent ethyl alcohol are presented in Table V.

All the quercetin compounds examined in this study could be easily separated except those of Band 2.1. The R_F value of Band 2.1 in several solvent systems is indicative of a monoglycoside. The sugars after hydrolysis of Band 2.1 were galactose and glucose, and the unhydrolyzed band

XUM

TABLE III

Identification of Aglycone Portion after Hydrolysis of Quercetin Components of Grimes Golden Apple Skin Extracts

Compound	R_F values in			Color reactions in		
	H_2O (9)*	*n*-BuOH-27 per cent aqueous HOAc (1:1) (5)*	PhOH-H_2O (73 per cent aqueous) (5)*	Ultraviolet light	Ultraviolet light + NH_3 vapor	Ultraviolet light + $AlCl_3$ spray (10)*
Aglycone from						
Band 1	0.01	0.78	0.34	Y.	Y.	Y.-G.
" 2.1	0.01	0.78	0.36	"	"	"
" 2.2	0.01	0.76	0.35	"	"	"
" 2.3	0.01	0.78	0.34	"	"	"
" 3	0.01	0.78	0.34	"	"	"
" 4	0.01	0.75	0.35	"	"	"
Quercetin	0.01	0.77	0.36	"	"	"

* Bibliographic reference.

TABLE IV

Identification of Sugars after Hydrolysis of Quercetin Components of Grimes Golden Apple Skin Extracts

Compound	R_F values in		Mm. movement in			Color aniline hydrogen oxalate (19)
	n-BuOH-benzene-pyridine-H_2O (5:1:3:3) (16)*	PhOH-H_2O (73 per cent aqueous) (5)*	EtOAc-HOAc-H_2O† (3:1:3) (17)*	*n*-BuOH-pyridine-H_2O‡ (3:1:1.5) (18)*	PhOH-H_2O (73 per cent aqueous) (5)*	
Sugar from						
Band 1				111	170	B.
" 2.1A				128	157	"
" 2.1B						
" 2.2	0.38	0.46	179			R.
" 2.3	0.30	0.56	156			"
" 3	0.51	0.62				B.
" 4	0.25	0.39				"
	0.50	0.61				"
Authentic sugars						
Glucose	0.25	0.40		128	158	B.
Galactose	0.23	0.46		111	171	"
Rhamnose	0.51	0.62	260			"
Arabinose	0.31	0.55	158			R.
Xylose	0.38	0.47	176			"
Lyxose	0.39	0.51				"
Ribose	0.45	0.64	222			"

* Bibliographic reference.

† Solvent allowed to run off end of paper.

‡ Development repeated three times (Jeanes *et al.* (7)).

contained no free sugar. After development (7) of Band 2.1 four times with *m*-cresol-acetic acid, a band 180 mm. wide was obtained. The leading and tailing 50 mm. of this band were cut out and eluted. When chromato-

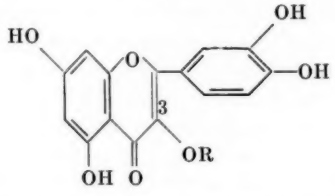
TABLE V

Maxima of Ultraviolet Absorption Spectra of Quercetin Components of Grimes Golden Apple Skin Extracts and Authentic Quercetin Compounds in 95 Per Cent Ethyl Alcohol

Compound	Band I, $\lambda_{max.}$	Band II, $\lambda_{max.}$
	mμ	mμ
Skin components		
Band 1	375	257
" 2.1	364	258
" 2.2	361	258
" 2.3	362	258
" 3	353	257
" 4	362	260
Authentic compounds		
Quercetin	373	257
Quercimeritrin	374	257
Hyperin	363	258
Isoquercitrin	360	258
Quercitrin	351	257
Rutin	361	258

TABLE VI

Quercetin Glycosides of Grimes Golden Apple Skin



Band No.	R	Compound
2.1A	Galactosyl	Hyperin
2.1B	Glucosyl	Isoquercitrin
2.2	Xylosyl	*
2.3	Arabinosyl	Avicularin
3	Rhamnosyl	Quercitrin
4	Rutinosyl	Rutin

* Not yet isolated in substance.

graphed with authentic samples, the leading and tailing eluates corresponded to hyperin and isoquercitrin, respectively, in both the *m*-cresol-acetic acid and the phenol solvents. It is concluded that Band 2.1 is composed of two monoglycosides of quercetin; *i.e.*, the 3-galactoside, hyperin, previously identified by Sando (2), and the 3-glucoside, isoquercitrin.

The presence of free quercetin in the apple skin was examined by an alternative extraction procedure. A 50 per cent hot methanolic extract of fresh Grimes Golden apple skin was prepared and subjected to paper chromatography. No free quercetin was present. It must be concluded that the aglycone quercetin does not exist in the apple skin, but is a hydrolysis product of the extraction procedure.

Identification—From the R_F values of the separated bands in several solvents, color reactions, identification of sugar and aglycone hydrolysis products, and ultraviolet absorption maxima, the bands were identified, as shown in Table VI.

DISCUSSION

The extract of the skin of Grimes Golden apples was found to contain quercetin and six quercetin glycosides. The preliminary ethyl ether and ethyl acetate extracts separated the aglycone and monoglycosides from the diglycoside. With water as the solvent the aglycone and glycosides were separated on the basis of the type and number or the absence of the sugar moiety. This relation was noted by Roberts and Wood (8, 9). A comparison of the R_F values of the skin components and of authentic compounds in several solvent systems served as one means of identification.

The color reactions of the spots of quercetin compounds on chromatograms in ultraviolet light, ultraviolet light plus ammonia vapor, and ultraviolet light after spraying with aluminum chloride are characteristics useful in identification of the compounds. When the 3 position is hydroxylated, as in quercetin and quercimeritrin, the color is yellow under ultraviolet light, yellow under ultraviolet light plus ammonia vapor, and yellow-green under ultraviolet light after spraying with aluminum chloride (10). The 3-glycosides of quercetin, such as quercitrin, avicularin, hyperin, isoquercitrin, and rutin, are brown to purple-brown under ultraviolet light, brown-yellow under ultraviolet light plus ammonia vapor, and yellow under ultraviolet light after spraying with aluminum chloride.

The ultraviolet absorption maxima of quercetin and its glycosides also indicated the nature of the substitution in the 3 position. The maximum of Band 1 of quercetin and quercetin glycosides with a free 3—OH group is 373 to 374 mμ. All the known 3-glycosides of quercetin except the rhamnoside quercitrin have their maximum between 360 and 363 mμ. The maximum of Band 1 for quercitrin is 351 mμ. Thus the position of the

maximum of Band 1 and the color reaction on the chromatogram together are of considerable diagnostic value.

Band 2.2 proved to be the 3-xyloside of quercetin, not previously reported. The presence of free xylose in apple juice and other fruits has been reported (11, 12). The other glycosides identified were hyperin, isoquercitrin, avicularin (13), quercitrin, and rutin. The complex of quercetin glycosides found in the Grimes Golden apple skin represents the largest number of glycosides related to a single aglycone reported from any plant tissue.

It was recently shown (14) that quercetin and some quercetin glycosides were not oxidized by tea oxidase. Similar results were obtained in this laboratory with a polyphenoloxidase preparation from apples. Therefore it is not probable that the quercetin compounds of apple skin serve as substrate for the browning reaction of apple scald.

SUMMARY

A procedure for the paper chromatographic separation and identification of a mixture of quercetin and quercetin glycosides is described. Hyperin (quercetin 3-galactoside), isoquercitrin (quercetin 3-glucoside), avicularin (quercetin 3-arabinoside), quercitrin (quercetin 3-rhamnoside), rutin (quercetin 3-rutinoside), and the hitherto unreported quercetin 3-xyloside were identified from the skin of Grimes Golden apples.

The author wishes to express his sincere appreciation to Dr. T. A. Geissman for his continued guidance and the provision of facilities during a portion of this investigation. I am grateful to Dr. E. C. Jorgensen for invaluable help and advice. I also wish to acknowledge the gifts of flavonoids from the following: Dr. T. A. Geissman, Dr. C. E. Sando, Dr. F. DeEds, Dr. S. H. Wender, and S. B. Penick and Company.

BIBLIOGRAPHY

1. Sando, C. E., *J. Agr. Res.*, **28,** 1243 (1924).
2. Sando, C. E., *J. Biol. Chem.*, **117,** 45 (1937).
3. Duncan, I. J., and Dustman, R. B., *J. Am. Chem. Soc.*, **58,** 1511 (1936).
4. Nordström, C. G., and Swain, T., *J. Chem. Soc.*, 2764 (1953).
5. Geissman, T. A., Jorgensen, E. C., and Johnson, B. L., *Arch. Biochem. and Biophys.*, **49,** 368 (1954).
6. Gage, T. B., and Wender, S. H., *Anal. Chem.*, **22,** 708 (1950).
7. Jeanes, A., Wise, C. S., and Dimler, R. J., *Anal. Chem.*, **23,** 415 (1951).
8. Roberts, E. A. H., and Wood, D. J., *Biochem. J.*, **49,** p. xxxiii (1951).
9. Roberts, E. A. H., and Wood, D. J., *Biochem. J.*, **53,** 332 (1953).
10. Gage, T. B., Douglass, C. D., and Wender, S. H., *Anal. Chem.*, **23,** 1582 (1951).
11. Kiyoshi, A., and Matsuda, K., *Tohoku J. Agr. Res.*, **2,** 135 (1951).
12. Hay, J. G., and Pridham, J. B., *Nature*, **172,** 207 (1953).

XUM

13. Ohata, T., *Z. physiol. Chem.*, **263,** 221 (1940).
14. Roberts, E. A. H., and Wood, D. J., *Nature*, **167,** 608 (1951).
15. Bate-Smith, E. C., in Williams, R. T., Partition chromatography, Biochemical Society symposia, Cambridge, **3,** 62 (1949).
16. Albon, N., and Gross, D., *Analyst*, **75,** 454 (1950).
17. Jermyn, M. A., and Isherwood, F. A., *Biochem. J.*, **44,** 402 (1949).
18. Chargaff, E., Levine, C., and Green, C., *J. Biol. Chem.*, **175,** 67 (1948).
19. Horrocks, R. H., and Manning, G. B., *Lancet*, **2,** 1042 (1949).

A PROTEOLYTIC INHIBITOR WITH ANTICOAGULANT ACTIVITY SEPARATED FROM HUMAN URINE AND PLASMA*

By N. RAPHAEL SHULMAN†

(From the United States Naval Medical Research Institute, Bethesda, Maryland)

(Received for publication, September 21, 1954)

The trypsin-inhibiting action of urine has been known for many years (1), but the inhibiting material has not been identified or isolated. In the present work a purified proteolytic inhibitor which differs from other known inhibitors was isolated from human urine, and some of its chemical and physical characteristics were studied. A similar inhibitor was obtained in less pure state from human plasma. Its action as an anticoagulant was of particular interest in considering the physiological implications of this inhibitor.

Of the proteolytic inhibitors that have been isolated from various sources, the inhibitor isolated from soy beans by Kunitz (2) is perhaps most extensively characterized. For this reason the soy bean inhibitor was compared with the new inhibitor in some of the reactions that were studied.

EXPERIMENTAL

Materials and Methods—Trypsin, α-chymotrypsin, and soy bean trypsin inhibitor were crystalline preparations obtained from the Worthington Biochemical Corporation. Protein concentration of each substance was determined by a micro-Kjeldahl technique and correlated with optical density measurements as done by Kunitz (2). For trypsin, with 15 per cent as the value for protein nitrogen, the conversion factor for protein concentration (mg. per ml.) from optical density was 0.636; for chymotrypsin, with 15.5 per cent protein nitrogen, the conversion factor was 0.51; for soy bean inhibitor, with 16.7 per cent protein nitrogen, the conversion factor was 1.14. The conversion factor obtained for trypsin was somewhat higher than the value of 0.585 obtained by Kunitz (2). The conversion factors of soy bean inhibitor and chymotrypsin were very close to those obtained by Kunitz.

Human plasma proteolytic enzymes were prepared from the euglobulin fraction of plasma obtained at low ionic strength, pH 5.2, and activated by

* This work was presented in part before the southern section meeting of the American Federation for Clinical Research, New Orleans, January 29, 1954.

† Lieutenant, Medical Corps, United States Navy, with the technical assistance of Jefferson W. Hamby, HM1, United States Navy, and William H. Sebrell, III.

chloroform or streptokinase (3). Streptokinase was supplied by the Lederle Laboratories Division, American Cyanamid Company. Bovine plasma proteolytic enzyme was obtained through the courtesy of Dr. E. C. Loomis.

The activity of trypsin and other enzymes was determined with the hemoglobin substrate of Anson (4). Incubation of enzyme and hemoglobin was done at 37° for 10 minutes with trypsin and chymotrypsin, and for 20 minutes with the other enzymes. The concentration of products of digestion was measured by optical density at 280 mμ. Enzyme activity is expressed in units according to Kunitz' definition (1 unit is the activity which produces an increase of 1 unit of optical density at 280 mμ per minute of digestion). Optical density was linearly proportional to enzyme concentration for the amounts of enzyme used. The activity of 1 γ of trypsin protein was 1.07×10^{-2} unit, and the combining proportion of trypsin with soy bean inhibitor was 1 part of trypsin to 0.84 part of inhibitor. The activity of 1 γ of chymotrypsin protein was 0.338×10^{-2} unit.

In several instances referred to in the text a fibrin substrate was used (5). The volume of the reaction mixture was the same as that with hemoglobin. Standard digestion curves for estimating activity were prepared as described previously (6). The amounts of enzyme used with fibrin are expressed in terms of their activity with hemoglobin.

Plasma for fractionation was obtained from the blood bank of the United States Naval Hospital, Bethesda, from routine donations which were considered unsuitable for transfusion. For coagulation studies fresh human plasma was obtained; 0.63 ml. of 4.0 per cent sodium citrate was used as an anticoagulant for 9 ml. of whole blood. Modifications of the recalcification and prothrombin time were used (7). The Chilcott thromboplastin was made up in one-half the recommended volume of solvent.

Sedimentation determinations were done in a Spinco, model E, analytical centrifuge with a 1.2 cm. cell. Sedimentation constants were corrected for the temperature and density of the medium (8).

Electrophoretic analyses of the inhibitor were carried out in a Klett apparatus with Longsworth scanning and a micro cell (2 ml.); those of the trypsin-inhibitor mixture in an Aminco apparatus, model B, with a semi-micro cell (4 ml.). In calculating per cent composition from electrophoretic patterns, it was assumed that all components had the same specific refractive increment.

The buffers used in electrophoretic and sedimentation determinations were those described by Miller and Golder (9) at 0.1 ionic strength. The buffer used in enzyme measurements was 0.06 M phosphate, pH 7.4. All pH values were determined with the Beckman glass electrode pH meter unless otherwise indicated.

Separation of Inhibitor from Urine—It was found that approximately 60 per cent of the total trypsin-inhibiting activity of normal urine was inactivated at 60° in 10 minutes in 2.5 per cent trichloroacetic acid. The remaining inhibiting activity was unchanged after 10 minutes at 80° in 2.5 per cent trichloroacetic acid, was non-dialyzable, and was precipitated by saturated ammonium sulfate and 85 per cent ethanol. The following procedure was used in purifying a trichloroacetic acid-soluble, heat-resistant inhibitor from urine.

Urine was obtained in pooled 24 hour collections from normal male adults. It was dialyzed in 20 to 40 liter batches in 2.5 inch cellulose tubing at room temperature for approximately 40 hours against vigorously stirred ammonium sulfate solution kept saturated with excess salt or was evaporated by vacuum distillation in 12 liter batches at approximately 35° for 24 hours. Urine volume was decreased approximately 5-fold by dialysis or evaporation, and an abundant precipitate was formed. The concentrated urine was saturated with solid ammonium sulfate and was kept at 5° for 1 or 2 days before the brown muddy precipitate was collected by centrifugation at 3000 × *g*. The precipitate from 1000 liters of urine was pooled.

The pooled precipitate was placed in cellulose tubing and dialyzed with vigorous agitation against running tap water for 2 days. The material remaining in the tubing was centrifuged at 3000 × *g* to remove coarse, undissolved particles and was then filtered with suction with several changes of Whatman No. 1 paper to remove any remaining precipitate. The filtrate, approximately 6 liters of clear dark brown liquid, was saturated with ammonium sulfate and allowed to stand for 24 hours at 5°. The precipitate which formed was collected on Whatman No. 44 paper with approximately 5 gm. of Celite (Johns-Manville) added per liter. The filtrate was discarded.

The precipitate containing Celite from the previous filtration was mixed with 300 ml. of water and filtered with suction on Whatman No. 44 paper to give a clear dark brown filtrate. The filtrate was brought to 85 per cent ethanol concentration at room temperature and the light brown precipitate that formed was collected with suction on Whatman No. 44 paper. The precipitate was washed with 85 per cent ethanol until the filtrate was colorless. The filtrate was discarded.

The precipitate was dissolved in 100 ml. of water and an equal volume of 5 per cent trichloroacetic acid was added. The mixture was allowed to stand at room temperature for 20 minutes, then heated to 80° for 10 minutes, cooled rapidly, and filtered with suction on Whatman No. 44 paper with several gm. of Celite. The precipitate was discarded. The crystal-clear amber-colored filtrate was brought to approximately pH 5 (indicator paper) with 2.5 N NaOH. It was then brought to 70 per cent ammonium

sulfate saturation by adding saturated ammonium sulfate solution and the precipitate that formed was collected on Whatman No. 44 paper with several gm. of Celite. The filtrate was discarded.

The light brown precipitate was mixed with 50 ml. of water and the insoluble material was removed on Whatman No. 44 paper. The filtrate was dialyzed with agitation against distilled water for 24 hours and the dialyzed solution was adjusted to pH 3.2 with 1.0 N HCl. A flocculent precipitate formed and increased at 5°. The precipitate was collected on hardened paper (Eaton-Dikeman No. 850) with suction at 5°, washed with several ml. of ice-cold water, then several ml. of acetone, and dried in air. 1.175 gm. of light tan powder capable of inhibiting a total of 315 mg. of trypsin were thus obtained.

Separation of Inhibitor from Plasma—2 liters of plasma were diluted with 19 volumes of distilled water at 5° and brought to pH 5.2 with 2 per cent acetic acid. The precipitate that formed was collected by centrifugation at 3000 × *g* and the supernatant fluid was discarded.

The precipitate was mixed with 200 ml. of 5 per cent trichloroacetic acid at room temperature and the mixture was churned vigorously for approximately 30 minutes until the insoluble material was in the form of small suspended particles which were then removed by centrifugation in a Servall centrifuge at 25,000 × *g* and discarded.

The supernatant fluid was brought to approximately pH 5.0 with 2.5 N NaOH, saturated with solid ammonium sulfate, and allowed to stand overnight at 5°. The white flocculent precipitate that formed was collected by centrifugation at 25,000 × *g* and washed with 50 ml. of cold saturated ammonium sulfate. The supernatant fluid was discarded.

The precipitate was dissolved in 15 ml. of water, and a small amount of insoluble material was removed on Whatman No. 44 paper with a thin layer of Celite. The crystal-clear filtrate was brought to 85 per cent ethanol concentration and the white flocculent precipitate that formed was collected on hardened paper (Eaton-Dikeman No. 850) with suction. The filtrate was discarded. The precipitate was dried in air and stored at 5° until the precipitate from 10 liters of plasma was accumulated.

The precipitate from 10 liters of plasma was dissolved in 25 ml. of water and dialyzed against distilled water for 24 hours with agitation. The dialyzed solution was brought to pH 3.2 with 0.1 N HCl and cooled to 5°. The white flocculent precipitate that formed was collected on hardened paper (Eaton-Dikeman No. 850) with suction at 5°, washed with several ml. of ice-cold water, then several ml. of acetone, and dried in air.

The precipitate was dissolved in water and adjusted to approximately pH 7.0 with 0.01 N NaOH. The clear colorless solution obtained was capable of inhibiting a total of 0.748 mg. of trypsin.

Results

The inhibitor prepared from urine and dried in air contained 59 per cent protein as determined by Kjeldahl nitrogen, assuming 16 per cent protein nitrogen. Amounts of this material used in subsequent experiments are expressed as protein weight. Ultraviolet absorption of an aqueous solution of the inhibitor prepared from urine is typical of proteins, a concentration of 1 mg. per ml. producing a density of 1.500 at 280 mμ (Fig. 1).

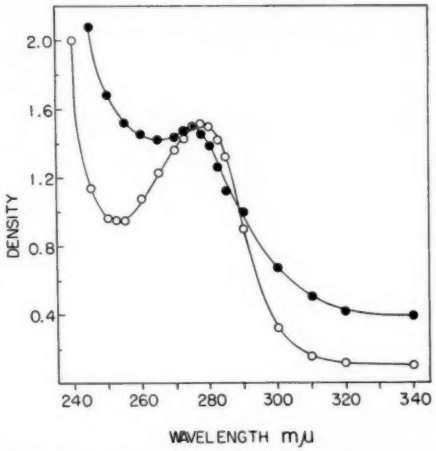


FIG. 1. Ultraviolet absorption spectra of the inhibitor from urine (○) and the inhibitor from plasma (●) at pH 7.0. Inhibitor from urine, 1.0 mg. per ml. Solution of inhibitor from plasma had 42.8 per cent of the trypsin-inhibiting activity of the solution of inhibitor from urine. 1 cm. light path, Beckman DU spectrophotometer.

Ultraviolet absorption of the inhibitor prepared from plasma is probably modified by the presence of impurities.

Effect of pH and Temperature on Inhibitor—At room temperature at pH 7.0 there was no loss in activity of the inhibitor for at least a week. There was no loss in activity when the inhibitor was incubated in 5 per cent trichloroacetic acid or 0.1 N HCl for 60 minutes or in 0.5 N HCl for 30 minutes at room temperature. The inhibitor was soluble in concentrations of trichloroacetic acid as high as 10 per cent at room temperature and in 2.5 per cent trichloroacetic acid at 80°. Incubation in 0.1 N NaOH at room temperature produced approximately 90 per cent loss of activity in 30 minutes and complete loss of activity in 45 minutes. Heating the inhibitor at 100° in phosphate buffer, pH 7.0, decreased activity approximately 70

per cent in 10 minutes and resulted in complete loss of activity after 20 minutes. Heating at 80° at pH 7.0 decreased activity approximately 30 per cent in 30 minutes and 60 per cent in 60 minutes. Heating at 80° in 2.5 per cent trichloroacetic acid produced the same decrease in activity as at pH 7.0. The loss of activity produced by heat at 100° or 80° was irreversible.

Reaction with Trypsin—Fig. 2 shows the stoichiometric nature of the combination of trypsin with the inhibitor from urine and plasma. Inhibition occurred as rapidly as the reagents could be mixed and was virtually irreversible. 1 γ of trypsin was inhibited by 2.2 γ of urine inhibitor. In subsequent experiments, amounts of plasma inhibitor are expressed as the weight of urine inhibitor with an equivalent activity against trypsin.

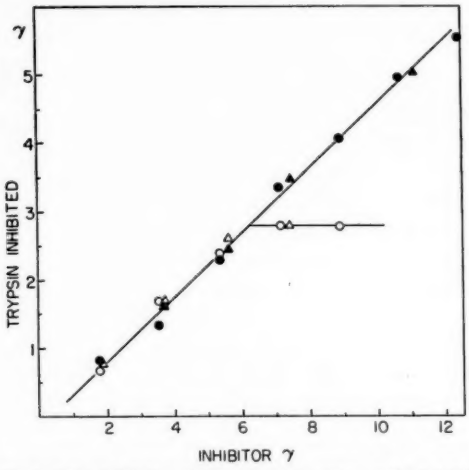


FIG. 2. The effect of the inhibitor from urine (●, ○) and the inhibitor from plasma (▲, △) on trypsin. Solid symbols, 5.6 γ of trypsin; open symbols, 2.8 γ of trypsin. 0.1 ml. of solution of inhibitor from plasma had trypsin-inhibiting activity equivalent to 3.74 γ of inhibitor from urine.

The inhibitor could be quantitatively recovered from the trypsin-inhibitor complex by treatment with 2.5 per cent trichloroacetic acid at 80° for 10 minutes. Trypsin precipitated and the inhibitor remained in the supernatant solution.

Reaction with Chymotrypsin and Plasma Enzymes—Fig. 3 shows the reversible type of inhibition obtained with the inhibitor from urine and plasma with chymotrypsin. The curves are calculated from Kunitz's equation for a reversible type of reaction (2). K, the equilibrium constant,

equal numerically to the value of inhibitor concentration at 50 per cent inhibition, was 0.058 mg. per ml. The amounts of urine and plasma inhibitor required to produce equivalent inhibition of chymotrypsin were the same.

Fig. 4 shows the type of inhibition observed with various plasma enzyme preparations. Curves are calculated, with 0.110 mg. as a K value for the bovine plasma enzyme, 0.375 mg. for the human plasma enzyme activated with streptokinase, and 1.210 mg. for the human plasma enzyme activated with chloroform. The amounts of urine and plasma inhibitor required to produce equivalent inhibition of the human plasma enzyme activated with

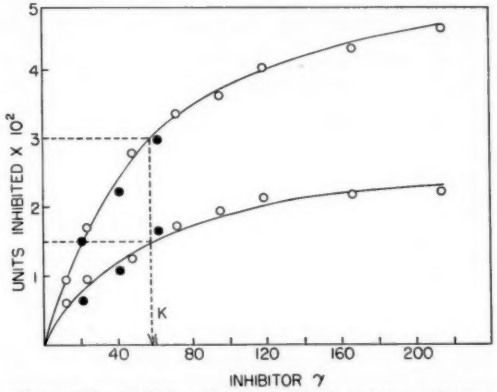


FIG. 3. The effect of the inhibitor from urine (○) and from plasma (●) on chymotrypsin. Upper curve, 6×10^{-2} unit of chymotrypsin; lower curve, 3×10^{-2} unit. Incubation time of inhibitor and enzyme 30 minutes at 22° before adding substrate. Value of K indicated by arrow.

streptokinase were the same. The other plasma enzymes were not tested with the inhibitor from plasma.

When a different substrate was used, the amount of inhibitor required to produce 50 per cent inhibition of a given amount of enzyme remained constant for trypsin but varied for the enzymes that had reversible reactions with the inhibitor. When fibrin was used as a substrate, the K value with 6×10^{-2} unit of chymotrypsin was 8.2 γ, with 1×10^{-2} unit of bovine plasma enzyme K was 13.0 γ, with 1×10^{-2} unit of human plasma enzyme activated with streptokinase K was 440 γ, with 1×10^{-2} unit of human plasma enzyme activated with chloroform K was 334 γ.

For comparison, the amount of soy bean inhibitor required for 50 per cent inhibition of the various enzymes with the two substrates was determined. With 6×10^{-2} unit of chymotrypsin the K value was 765 γ with

the hemoglobin substrate and 6.6 γ with the fibrin substrate. With 1×10^{-2} unit of bovine plasma enzyme the K value was 5.5 γ with hemoglobin and 6.8 γ with fibrin. With 1×10^{-2} unit of human plasma enzyme activated with streptokinase the K value was 3.9 γ with hemoglobin and 8.4 γ with fibrin; with 1×10^{-2} unit of human plasma enzyme activated with chloroform the K value was 2.1 γ with hemoglobin and 0.51 γ with fibrin.

Effect on Blood Coagulation—The effect of different amounts of the inhibitor from urine and plasma on the clotting time of recalcified plasma is recorded in Table I. Similar amounts of each inhibitor preparation

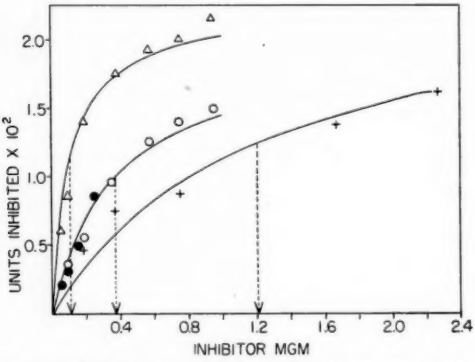


FIG. 4. The effect of the inhibitor from urine on 2.25×10^{-2} unit of bovine plasma enzyme (△), on 2.0×10^{-2} unit of human plasma enzyme activated with streptokinase (○), and on 2.5×10^{-2} unit of human plasma enzyme activated with chloroform (+). The effect of the inhibitor from plasma on 2.0×10^{-2} unit of human plasma enzyme activated with streptokinase (●). Incubation time of inhibitor and enzymes 30 minutes at 22° before adding substrate. Values of K indicated by arrows.

(from urine or plasma) produced similar prolongation of clotting time. Inhibition of coagulation was proportional to the trypsin-inhibiting activity at each stage of purification of the inhibitor and could be abolished by neutralization of the inhibitor with trypsin. Subsequent experiments on coagulation were done with the inhibitor from urine.

A comparison of the prolongation of the prothrombin time and the recalcification time of plasma by the inhibitor from urine is shown in Table II. It is known that the recalcification time at this dilution of plasma does not become prolonged until prothrombin concentration is decreased to about 20 per cent or less (7). With 25 γ of inhibitor, the recalcification time was prolonged, although the apparent concentration of prothrombin was still 62 per cent. This finding suggested that a decrease in prothrombin was not the cause of changes in clotting time, but that there was some

other cause for a decrease in thrombin formation. Thromboplastin in excess of the optimal amount did not alter the effect of the inhibitor on the prothrombin time. The inhibitor was found to have no direct effect on thrombin when as much as 6.5 mg. were mixed with 2 units of purified thrombin (Parke, Davis and Company).

The same type of inhibition was observed when soy bean inhibitor was used in place of the urine inhibitor. The weight of soy bean inhibitor required to produce an equivalent effect was approximately 0.16 the weight

TABLE I

Comparison of Anticoagulant Action of Inhibitor from Urine and Plasma

The reaction mixture contained 0.3 ml. of citrated plasma, varying amounts of inhibitor, and sufficient 0.85 per cent NaCl to give a final volume of 1.8 ml. Recalcification was done with 0.02 ml. of 3.0 per cent $CaCl_2$. Clotting time at 37° taken as the time between the addition of Ca^{++} and the formation of a clot firm enough to be inverted in a 7 mm. tube.

Inhibitor from urine*	Inhibitor from plasma†	Clotting time
γ	γ	*sec.*
0	0	350 ± 50
220	0	380
440	0	630
660	0	1420
0	202	340
0	404	540
0	606	1130

* 0.10 ml. of urine inhibitor = 220 γ.

† Trypsin-inhibiting activity of 0.5 ml. of plasma inhibitor equivalent to 202 γ of urine inhibitor.

of the inhibitor from urine. The soy bean inhibitor and the urine inhibitor were additive in their effects on coagulation.

Sedimentation of Inhibitor—Fig. 5, *A* shows the sedimentation pattern of the inhibitor preparation from urine. The material showed characteristics of a homogeneous protein in that the sedimentation curve had a single peak with only slight asymmetry. The sedimentation constants at different concentrations of inhibitor, shown in Fig. 6, gave a value of $s_{20,w} = 2.04$ S. A diffusion constant was calculated from the area and height (10) of sixteen sedimentation curves obtained at four different concentrations of inhibitor. The mean $D_{20,w}$ of the individual determinations was 12.05×10^{-7} cm.^{2} sec.$^{-1}$ with a 2Σ variation of 19 per cent. From the above sedimentation and diffusion constants, and the assumption of a partial specific

volume of 0.75 cm.³ per gm., the calculated molecular weight of the inhibitor was 16,400.

Electrophoresis of Inhibitor—Fig. 7, *A* shows the electrophoretic pattern of the inhibitor preparation from urine. Two components were present from pH 1.9 to 7.05. A slow moving component (I_1) comprised 78 per cent of the material; a faster moving component (I_2) 22 per cent. The mobilities of the components at different pH values are shown in Fig. 8. The isoelectric point of each is near pH 2.8.

TABLE II

Comparison of Effects of Inhibitor on Prothrombin Time and Recalcification Time

The prothrombin time reaction mixture contained 0.1 ml. of citrated plasma, varying amounts of inhibitor, 0.05 ml. of thromboplastin, and sufficient 0.85 per cent NaCl to give a final volume of 0.23 ml. The recalcification time reaction mixture contained 0.1 ml. of citrated plasma, varying amounts of inhibitor, and sufficient 0.85 per cent NaCl to give a final volume of 0.23 ml. Recalcification in all tubes done with 0.02 ml. of 1.0 per cent $CaCl_2$. Temperature 37°. Apparent concentration of prothrombin read from curve of clotting time with serial saline dilutions of same sample of plasma.

Inhibitor from urine*	Prothrombin time		Recalcification time
	Clotting time	Apparent concentration of prothrombin	Clotting time
γ	*sec.*	*per cent*	*sec.*
0	16.6–17.2	100	350 ± 20
25	20.1	62	450
50	23.3	42	590
100	29.3	26	750
200	36.0	17	960
300	40.5	14	1780
400	49.0	11	1760

* 0.01 ml. of inhibitor = 50 γ.

Electrophoresis of Mixture of Inhibitor and Trypsin—Trypsin was combined with the urine inhibitor, 2.2 parts by weight of inhibitor to 1 part of trypsin. 1 ml. of a 1.2 per cent solution of this mixture was found to have proteolytic activity equivalent to 35 γ of trypsin. This indicated over 99 per cent inactivation of trypsin. The electrophoretic pattern of the mixture at pH 7.05 is shown in Fig. 7, *B* and the mobilities of the different peaks in Fig. 8. The largest peak ($T + I$) did not have the mobility of trypsin (11) or of either of the components of the inhibitor. The next largest peak (I_1) had the mobility of the slow component of the inhibitor, and the smallest peak (I_2) had the mobility of the fast component of the inhibitor. At pH 5.15 the same peaks were present, but the largest peak

moved toward the cathode. The other peaks moved toward the anode and again had the same mobility as the peaks of the plain inhibitor. The material in the largest peak had an isoelectric point of approximately pH 6.20, which is between that of the inhibitor and that of trypsin (11). It appeared probable that this was the trypsin-inhibitor complex and that the other components were material in the inhibitor preparation that did not combine with trypsin. The trypsin-inhibitor complex was calculated to

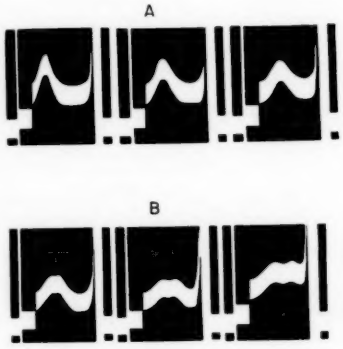


FIG. 5

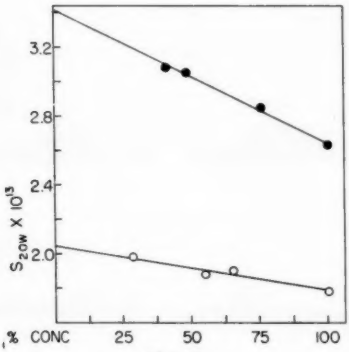


FIG. 6

FIG. 5. *A*, sedimentation pattern of inhibitor, 1.1 per cent protein, pH 7.0. Average speed, 56,000 r.p.m.; average temperature, 22.6°. From left to right, 96, 128, 160 minutes after first exposure. *B*, sedimentation pattern of inhibitor and trypsin mixture, 1.2 per cent protein, pH 7.05. Average speed, 56,000 r.p.m.; average temperature, 25.8°. From left to right, 96, 128, 160 minutes after first exposure.

FIG. 6. Sedimentation constants of the inhibitor (○) and the trypsin-inhibitor complex (●). 100 per cent concentration of the inhibitor = 0.9 per cent protein; 100 per cent concentration of the trypsin-inhibitor complex = 0.64 per cent protein; the total protein concentration of the mixture of trypsin and inhibitor = 1.2 per cent. pH 7.0.

be 53.3 per cent of the total material present. Since practically all of the trypsin was combined with inhibitor and the proportion of trypsin to inhibitor in the mixture was 1.0:2.2, the ratio of trypsin to inhibitor in the actual complex would be 1.00:0.71. If the molecular weight of trypsin is 24,000 (12), the molecular weight of the inhibitor, in a 1:1 combination, would be 17,000.

It was possible to determine from the electrophoretic patterns which of the two components in the inhibitor preparation combined with trypsin. The calculation was made from the per cent composition of the components of the plain inhibitor and of the mixture of trypsin and inhibitor. The larger component of the inhibitor (I_1 in Fig. 7) accounted for 79 per cent of

the total of the plain inhibitor. The larger component of the uncombined inhibitor in the mixture of inhibitor and trypsin accounted for only 47 per cent of the total inhibitor used. The change in the amount of the larger component of the inhibitor after mixture with trypsin was equivalent to 32 per cent of the total inhibitor and the amount of inhibitor that combined

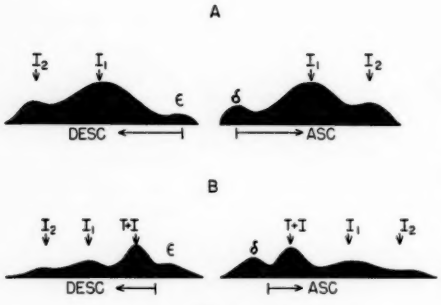


FIG. 7

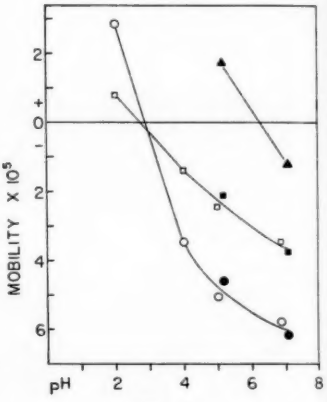


FIG. 8

FIG. 7. *A*, electrophoretic pattern of inhibitor, pH 6.85, 1.05 per cent protein, after 9000 seconds. The two components are indicated by I_1 and I_2. *B*, electrophoretic pattern of the mixture of inhibitor and trypsin, pH 7.05, 1.2 per cent protein, after 4200 seconds. The trypsin-inhibitor complex indicated by $T + I$; the two components of the uncombined inhibitor by I_1 and I_2.

FIG. 8. Influence of pH on the electrophoretic mobility of the components of the inhibitor and of the mixture of inhibitor and trypsin (depicted in Fig. 7). The values are the average of the ascending and descending patterns. First component (I_1) of the inhibitor in the plain inhibitor (□) and in the mixture of inhibitor and trypsin (■). Second component (I_2) of the inhibitor in the plain inhibitor (○) and in the mixture of inhibitor and trypsin (●). Trypsin-inhibitor complex ($T + I$) (▲).

with trypsin was 32 per cent of the total inhibitor (see above). The active inhibiting substance was therefore in the larger component of the inhibitor and comprised 41 per cent of that component.

Sedimentation of Trypsin-Inhibitor Mixture—The same mixture of inhibitor and trypsin used for Fig. 7, *B* gave the sedimentation pattern shown in Fig. 5, *B*. Two components of about equal magnitude were present. The sedimentation constant of the slower moving component was the same as that of the plain inhibitor. The faster moving component was the trypsin-inhibitor complex. The sedimentation constants of the trypsin-inhib-

itor complex at different concentrations are shown in Fig. 6. A value of $s_{20,w} = 3.42$ S was obtained.

DISCUSSION

The trypsin-inhibiting activity of the total yield of purified inhibitor from urine was approximately 5 to 10 per cent of the total activity in the original urine that was processed. The trypsin-inhibiting activity of whole urine which is inactivated at 60° at 2.5 per cent trichloroacetic acid concentration was eliminated in the purification procedure.

The finding that the inhibitor preparation appeared homogeneous on sedimentation and heterogeneous on electrophoresis corresponds to similar observations on the ovomucoid inhibitor (13). From electrophoretic analysis it was found that the larger component of the inhibitor preparation contained the active substance. Since only 41 per cent of that component combined with trypsin, it was assumed that the remainder had lost its trypsin-inhibiting activity, although it still had the molecular characteristics of the active inhibitor. Apparently, successive treatment with the various reagents used led to approximately 60 per cent loss in activity of the inhibitor.

The inhibitor from plasma is probably identical with the inhibitor from urine. The inhibitor from each source was purified under the same general conditions and had the same properties of stability. In each case the reaction with trypsin was the same, and the same amounts were required for inhibition of chymotrypsin, bovine plasma enzyme, and blood coagulation. It is not possible to estimate precisely the amount of this type of inhibitor that is present in plasma. The inhibitor that was purified differs from the inhibitor that is present in high concentration in plasma since only about 3 per cent of the trypsin-inhibiting activity of plasma remains after plasma is treated with 2.5 per cent trichloroacetic acid (14) and only about 5 per cent of the activity remains after diluted plasma is heated at 60° (15). Whole plasma is capable of inhibiting approximately 2 mg. of trypsin per ml. (15) and the quantity of purified inhibitor obtained per ml. of plasma had approximately 0.004 per cent of this activity. It is possible that much of the inhibitor was lost by the method of purification used. A number of other methods of purification were tried in an attempt to increase the yield. Various fractions of whole plasma obtained with different concentrations of alcohol, acid, and ammonium sulfate were used as the starting material, but none gave a satisfactory yield except the euglobulin fraction. This fraction was used since it was known that it contained the plasma proteolytic enzymes as well as many of the clotting factors (16), and it was considered that the inhibitor might precipitate in association with the substances with which it reacts. It is of interest that, after the inhibitor was

removed from the euglobulin fraction by acid extraction, the remainder of the fraction showed proteolytic activity similar to that obtained when the euglobulin fraction was treated with chloroform for proteolytic enzyme activation. The inhibitor may be present in the euglobulin fraction in combination with proteolytic enzymes.

The purified inhibitor had characteristics suggesting that it was similar to the inhibitor obtained from bovine plasma by Schmitz (17). Three preparations of human plasma inhibitor were made following Schmitz's technique and in each instance the total inhibiting activity recovered was approximately 10 per cent of the activity obtained from the same amount of plasma by the method described above. The inhibitor prepared by Schmitz's method was found to have the same heat stability, solubility in trichloroacetic acid, and properties of precipitation in ethanol and ammonium sulfate as the present inhibitor. Schmitz did not observe any inhibition of chymotrypsin with the amounts of inhibitor he used. However, it was found in this laboratory that Schmitz's inhibitor did show a reversible reaction with chymotrypsin and that equal amounts of his inhibitor and the present inhibitor were required for 50 per cent inhibition of chymotrypsin. The largest amount of inhibitor he used with chymotrypsin apparently was insufficient to produce detectable inhibition. It was found in this laboratory that the amount of inhibitor obtained from 3 liters of human plasma by Schmitz's method was insufficient to cause prolongation of the recalcification time of 0.3 ml. of plasma, but Grob (18) obtained an inhibitor by Schmitz's method using 5 liters of pig blood and was able to demonstrate inhibition of coagulation. It appears that the inhibitor prepared by Schmitz and the present inhibitor may be identical.

The combination of trypsin and the purified inhibitor was irreversible as far as could be determined with the substrates used and by electrophoretic and sedimentation analysis of the trypsin-inhibitor complex in the range pH 5.15 to 7.05.

The combination of the inhibitor with chymotrypsin and plasma enzymes was readily reversible when a hemoglobin substrate was used. With chymotrypsin and the bovine plasma enzyme the equilibrium constant was markedly lower when a fibrin substrate was used. The equilibrium constant was so low in the case of the fibrin substrate that inhibition appeared to be directly proportional to the amount of inhibitor added over a wide range of inhibitor concentration, thus giving the appearance of an irreversible reaction. A similar change in the equilibrium constant of chymotrypsin with the soy bean inhibitor was observed when the two different substrates were used. There have been differences in opinion concerning whether the inhibition of chymotrypsin by plasma is a reversible or irreversible type of reaction (14, 6). The fact that different substrates were used in measuring inhibition may account for these differences in opinion.

A comparison of the effect of the inhibitor on the prothrombin time and recalcification time (Table II) indicated that the decrease in thrombin formation was not due to an effective decrease in available prothrombin. The inhibitor also did not act on thrombin or thromboplastin. It was found that the inhibitor from urine and the soy bean inhibitor had a similar anticoagulant effect. Glendening and Page (19), working with soy bean inhibitor and purified prothrombin, found that the inhibitor did not act on thrombin, thromboplastin, or accelerator globulin, but that it did decrease the rate of thrombin formation as well as the final yield of thrombin. The work of Glendening and Page was repeated in this laboratory and confirmed with respect to both the soy bean and urine inhibitors. They had concluded that the inhibitor forms a dissociable complex either with prothrombin or a derivative of prothrombin. Our results support the view that the inhibitor does not act directly on prothrombin but on an intermediate product between prothrombin and thrombin.

Amounts of the soy bean inhibitor and the inhibitor separated from urine and plasma which inhibit as much as 0.1 mg. of trypsin also markedly inhibit blood coagulation. Approximately 0.05 ml. of whole plasma itself is capable of inhibiting 0.1 mg. of trypsin, and yet prothrombin-free plasma added in much greater amounts than this to 0.1 ml. of whole plasma does not inhibit coagulation (20). The level of plasma inhibition of trypsin may become elevated to 2 or 3 times normal in various disease states without an associated coagulation defect (15). The inhibitors which account for most of the trypsin-inhibiting activity of plasma apparently do not act as anticoagulants. If the indications of the concentration of the purified inhibitor in plasma described above are correct, it would not be present in sufficient amount to affect coagulation under normal conditions. Whether or not such an anticoagulant plays a rôle in normal or abnormal coagulation phenomena remains to be investigated.

The molecular weight of the inhibitor obtained from experimentally determined sedimentation and diffusion constants was 16,400, and the molecular weight obtained by electrophoretic analysis of the combination of inhibitor with trypsin was 17,000. By using the average of the two (16,700) as the molecular weight, a calculated diffusion constant for an unhydrated sphere of 12.6×10^{-7} cm.^{2} sec.$^{-1}$ was obtained. The diffusion constant obtained experimentally was 12.05×10^{-7} cm.^{2} sec.$^{-1}$ and the ratio of the two gave a dissymmetry constant of $f/f_0 = 1.05$ which is a plausible value. On the basis of a molecular weight of 40,700 (24,000 + 16,700) for the trypsin-inhibitor complex, the sedimentation constant obtained from a calculated diffusion constant for an unhydrated sphere was $s_{20,w} = 4.3$ S. The ratio of this sedimentation constant to the one obtained experimentally (3.42) gave a dissymmetry constant of $f/f_0 = 1.26$ which is a plausible value. It appears from a comparison of the experimentally determined

diffusion and sedimentation constants with those calculated from the molecular weight alone that 16,700 is a reasonably accurate molecular weight for the inhibitor. To correct for impurities in the inhibitor preparation, the amount of inhibitor used in the above experimental work should be multiplied by 0.316.

SUMMARY

1. The methods for purifying a proteolytic inhibitor with anticoagulant activity from human urine and plasma were described.

2. The molecular weight of the inhibitor was found to be approximately 16,700. The isoelectric point of the inhibitor was pH 2.8 and that of the trypsin-inhibitor complex was approximately pH 6.2.

3. The inhibitor was relatively resistant to denaturing conditions.

4. The reactions of the inhibitor with trypsin, chymotrypsin, and various plasma proteolytic enzymes were studied and were compared with those of the soy bean trypsin inhibitor.

5. The combination of the inhibitor with trypsin was virtually irreversible at near neutral pH. The inhibitor could be recovered from the trypsin-inhibitor complex by treatment with 2.5 per cent trichloroacetic acid.

6. The reaction of the inhibitor with chymotrypsin and various plasma proteolytic enzymes was reversible.

7. The inhibitor had an anticoagulant activity similar to that of the soy bean trypsin inhibitor. It appeared to act on an intermediate product between prothrombin and thrombin.

I wish to express my appreciation to Dr. Robert F. Steiner for his suggestions and criticisms and for the ultracentrifuge experiments, and to Dr. Maxime MacKenzie and to Dr. Harry A. Saroff for the electrophoretic experiments.

BIBLIOGRAPHY

1. Bauer, J., and Reich, Z., *Biochem. Centr.*, **9**, 510 (1909).
2. Kunitz, M., *J. Gen. Physiol.*, **30**, 291 (1946).
3. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, **28**, 559 (1945).
4. Anson, M. L., *J. Gen. Physiol.*, **22**, 79 (1938).
5. Shulman, N. R., and Tagnon, H. J., *J. Biol. Chem.*, **186**, 69 (1950).
6. Shulman, N. R., *J. Exp. Med.*, **95**, 571 (1952).
7. Quick, A. J., The hemorrhagic diseases, Springfield (1942).
8. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
9. Miller, G. L., and Golder, R. H., *Arch. Biochem. and Biophys.*, **29**, 420 (1950).
10. Greenberg, D. M., Amino acids and proteins, Springfield (1951).
11. Bier, M., and Nord, F. F., *Arch. Biochem. and Biophys.*, **33**, 320 (1951).
12. Cunningham, L. W., Jr., Tietze, F., Green, N. M., and Neurath, H., *Discussions Faraday Soc.*, **13**, 58 (1953).

XUM

13. Fredericq, E., and Deutsch, H. F., *J. Biol. Chem.*, **181**, 499 (1949).
14. Peanasky, R. J., and Laskowski, M., *J. Biol. Chem.*, **204**, 153 (1953).
15. Shulman, N. R., *J. Exp. Med.*, **95**, 593 (1952).
16. Mellanby, J., *Proc. Roy. Soc. London, Series B*, **107**, 271 (1930).
17. Schmitz, A., *Z. physiol. Chem.*, **255**, 234 (1938).
18. Grob, D., *J. Gen. Physiol.*, **26**, 423 (1943).
19. Glendening, M. B., and Page, E. W., *J. Clin. Invest.*, **30**, 1298 (1951).
20. Rosenfield, R. E., and Tuft, H. S., *Am. J. Clin. Path.*, **17**, 405 (1947).

DETERMINATION OF TOTAL CARBON AND ITS RADIOACTIVITY

II. REDUCTION OF REQUIRED VOLTAGE AND OTHER MODIFICATIONS*

By F. MAROTT SINEX, JOHN PLAZIN, D. CLAREUS, W. BERNSTEIN, DONALD D. VAN SLYKE, AND R. CHASE

(From the Department of Medicine and the Department of Instrumentation and Health Physics, Brookhaven National Laboratory, Upton, New York)

(Received for publication, September 7, 1954)

Radioactive carbon has been routinely determined in this laboratory by gas phase proportional counting (1). In this procedure the total CO_2 is first measured in the Van Slyke-Neill manometric apparatus, then transferred to the counting tube where methane is added until the CO_2-methane mixture reaches atmospheric pressure. The counting is done with a potential of 3800 volts across the counter. Approximately 98 per cent of the disintegrations occurring within the volume enclosed by the silvered area of the counter are measured (1–3). The presence of more than 16 per cent of CO_2 in the methane-CO_2 mixture depresses the count, but with the use of a correction curve (Fig. 10 (1)) mixtures containing up to 30 per cent CO_2 may be counted with little sacrifice of accuracy.

In the present paper, a modification is presented which permits counting at approximately 2000 volts. The $C^{14}O_2$ is mixed with a gas consisting of 90 per cent argon and 10 per cent methane, commonly called "P-10."[1] The use of the lower voltage has some advantages. Operation at 2000 volts is less likely to give trouble than operation at 3800 volts, particularly in moist weather in a room lacking humidity control, and is therefore likely to require less frequent servicing. Furthermore, well regulated power supplies delivering 2500 volts are more generally available than those delivering 4000 to 5000 volts. The only disadvantage encountered in the use of the P-10 gas in place of methane is that the maximal size of carbon sample that can be used with the P-10 is about 6 mg. of carbon, compared with 16 mg. with the methane. This difference is seldom important, since samples of over 4 mg. of carbon are required only in rare cases, when the specific C^{14} activity is unusually low (see Fig. 12 (1)).

Electronic Apparatus

The electronic instrumentation used in this procedure consists of a high voltage supply, amplifier, discriminator, and scaling circuit. A block dia-

* This research was supported by the Atomic Energy Commission.

[1] The argon-methane "P-10" mixture is commercially available from the Matheson Company, Inc., East Rutherford, New Jersey.

gram of this assembly is presented in Fig. 1. The high voltage supply is stable to 0.1 per cent for long time periods. The amplifier has a maximal input sensitivity of 0.3 mv. and is not subject to spurious counts or paralysis from overloading of the amplifier by large events when the gain is sufficiently high to detect the small events.[2] The discriminator has a fixed 5 volt sensitivity (responds to pulses of more than 5 volts) and delivers a 20 volt negative pulse, which is suitable for most scalers.

Fig. 2 is a circuit diagram of the amplifier and discriminator. The amplifier we have used has three fixed input sensitivities, *viz.* 0.3, 10, and 300 mv. The 0.3 mv. input is used for the proportional counter of the present method. (The 10 mv. input is suitable for scintillation counters, the 300 mv. input for Geiger counters.)

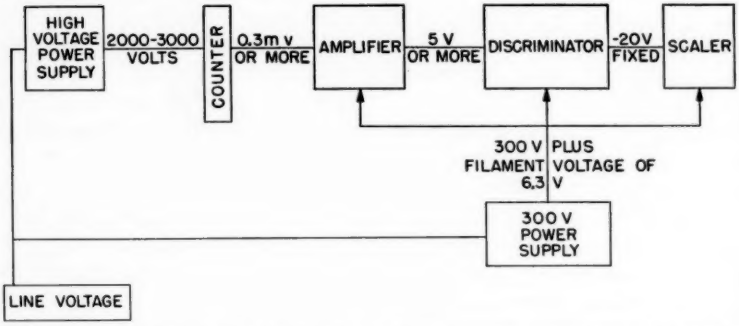


FIG. 1. A simplified circuit diagram of the equipment required to count $C^{14}O_2$ in 90 per cent argon, 10 per cent methane.

As can be seen in Fig. 2, 300 volts and a filament supply are necessary for operation of the amplifier and discriminator. We have not indicated the sources of these voltages in the circuit diagram since they may be derived from several sources. In our present arrangement these voltages are derived from a commercial scaling circuit and power supply combination, which also provides the high voltage for the counter. In such a combination, however, some problems arise from the introduction of switching transients in the power lines, with consequent spurious counts. The particular unit which was used was somewhat cramped and difficult to construct because it was desirable to have the amplifier fit into the scaler box. A simpler procedure is to construct the amplifier with its own 300

[2] In the previous paper (1) an amplifier with an input sensitivity of 1 mv. for the methane-CO_2 counter was specified. The only essential change from the previous specifications (1) is increase in input sensitivity to 0.3 mv. The apparatus here described can be used with either methane or P-10 counters. For a discussion of the design of amplifiers of this type see Higinbotham (4).

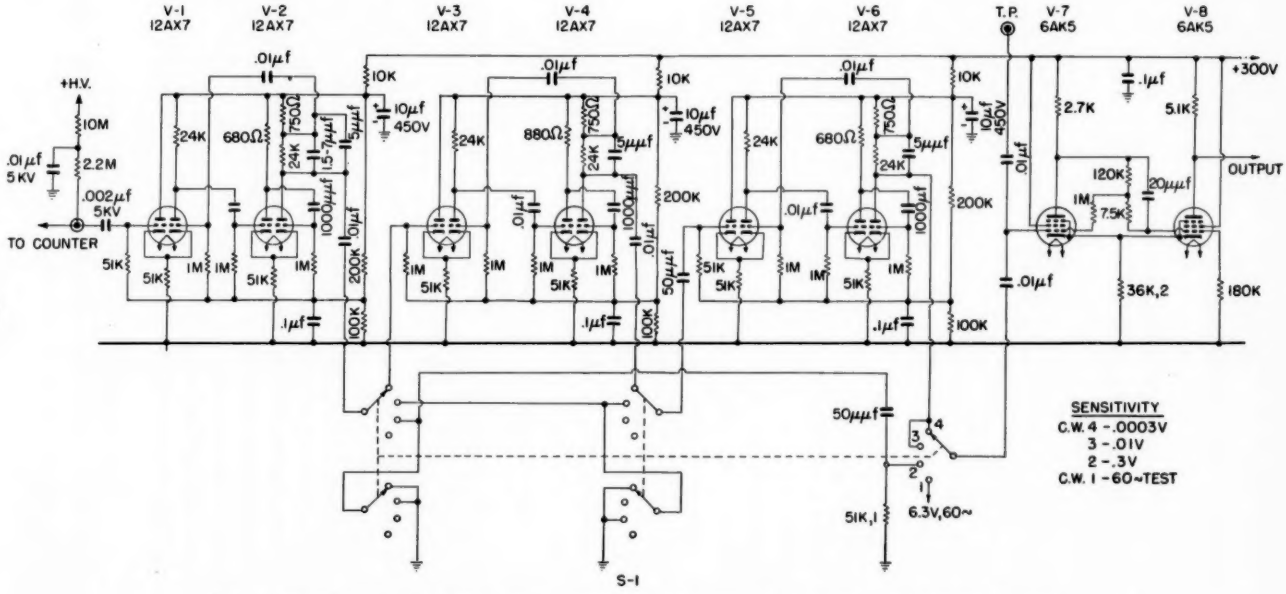


FIG. 2. Circuit diagram of the pulse amplifier that has been found well suited to C^{14} counting

volt and filament supplies (such supplies are commercially available) as a completely separate unit.

EXPERIMENTAL

In order to determine the proportion of disintegrations counted in an argon-methane-CO_2 mixture, a constant amount of C^{14} in the form of $Na_2C^{14}O_3$ was mixed with varying proportions of $Na_2C^{12}O_3$ and analyzed as described on pp. 793–795 of the paper by Van Slyke, Steele, and Plazin

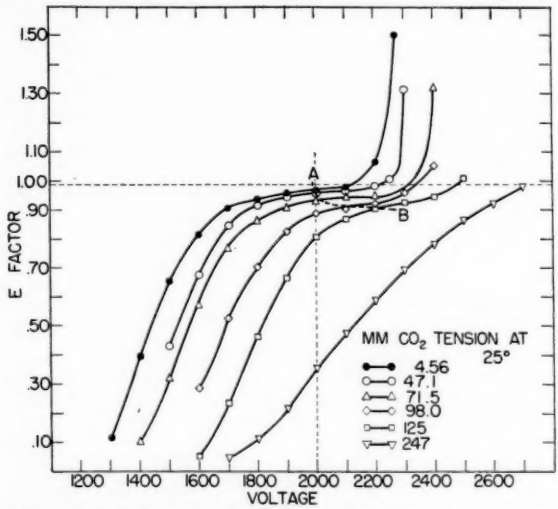


FIG. 3. Relation of $C^{14}O_2$ count to applied voltage in gas phase counter. Each curve shows results with an indicated concentration of total CO_2 present in the CO_2-argon-methane mixture. Factor E is defined in the text.

(1), except that the 90:10 argon-methane mixture instead of methane was used in the present work. The $C^{14}O_2$ was kept constant at an amount to give at 3800 volts approximately 70,000 c.p.m. in a methane-CO_2 mixture, with less than 16 per cent of total CO_2. The same count, within the limits of error, was given by the same amount of $C^{14}O_2$ in the present experiments with P-10-CO_2 mixtures containing less than 6 per cent of total CO_2, or 45 mm. of CO_2 tension.

The results of the present experiments are presented in Fig. 3. The ordinates represent counts per minute in terms of the efficiency factor E, calculated as

$$E = 0.98 \times \frac{\text{counts per minute observed in P-10 gas}}{\text{counts per minute observed in methane}}$$

(see (1), pp. 793–795).

Except for the curve with the highest CO_2 tension (247 mm., 30 per cent CO_2), each curve shows a count rising with the voltage to an approximately level plateau, where presumably the maximal number of disintegrations with energies within the sensitivity range of the apparatus is counted. Higher voltages cause a rapid rise of the curve above the count level that corresponds to C^{14} disintegrations.

In practice it has been found satisfactory to use a voltage about one-third the length of the plateau from its upper voltage limit. Such a point is at about 2000 volts for gas mixtures with under 70 mm. of CO_2 tension (9 per cent CO_2), but is definitely at higher voltages for higher tensions, as indicated by the intersections of the dash curve *A-B* with the plateau curves.

For analyses with less than 5 mg. of carbon (CO_2 tensions under 80 mm.), a voltage of 2000 serves with the counter tubes that we have used. However, curves of the type of Fig. 3 can vary somewhat with the diameter and properties of the anode wire and with the properties of the amplifier-discriminator unit employed. For the most accurate work, it is desirable to determine the voltage *versus* count curve with each counter tube used with the approximate amount of CO_2 in the carbon samples usually employed (*e.g.* 2 to 3 mg. of C), and from the observed curve estimate the voltage that will lie on the counter's plateau a little nearer the upper than the lower end of the plateau.

In Fig. 4 are given the *E* factors obtained with three different tubes and with carbon samples up to 16 mg.; all the tubes were operated with a constant 2000 volt potential across the counter. It is seen that, as the carbon sample rises above 6 mg., the *E* factor falls rather rapidly (for the P-10 gas) and also shows more variation from one counter tube to another. Hence for use with the P-10 gas, it is not desirable as a rule to use samples with more than 6 mg. of carbon, giving 100 mm. of CO_2 pressure in 100 ml. counter tubes. In practice, for reasons stated in the introduction, this limitation is seldom significant. For most accurate activity calculations, one should prepare one's own version of Fig. 4, covering the area for carbon samples below 6 mg. quantitatively, as in Fig. 10 of the previous paper (1). The *E* values plotted are used for calculation of millimicrocuries by Equation 6 of the previous paper (1). For samples of less than 3 mg. of carbon, *E* can be taken as 0.98 without significant error. For samples of more than 6 mg. the *E* factor becomes so variable that it is not advisable to use such samples with the argon-methane mixture.

Widened First Limb of Freezing Coil

A slight change is advisable in the construction of the connecting tube (*D* of Fig. 6 (1)) in the gas apparatus described in the previous paper. The tube has a coil for immersion in dry ice-alcohol to freeze water vapor.

During summer weather, with high room temperature and consequent high vapor pressure of water in the manometric chamber, the amount of water vapor trapped has been so great that it has sometimes formed an ice plug across the 8 mm. tube which forms the first descending limb of the glass coil. Closing of the tube by such an ice plug has occurred in only a few of several hundred analyses. To prevent the occlusion it is

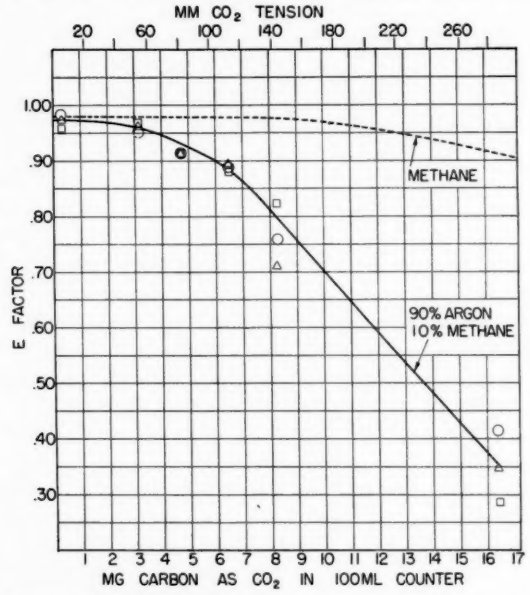


FIG. 4. Efficiency factor, E, at 200 volts, of gas counter with carbon samples up to 16 mg. Results with three counter tubes, D-1, D-2, and D-3, indicated by symbols ○, △, □, respectively, are given for the argon-methane-CO_2 mixture. The dash curve gives results with methane-CO_2 mixtures (*cf.* (1)).

advisable to make the first descending limb of the coil 10 or 11 mm. in external diameter instead of 8 mm. Also, it is advisable, especially in warm weather, to dry the connecting tube after each analysis by drawing through it acetone, followed by air.

Preparation of NaOH-Hydrazine Solution of Minimal CO_2 Content for Use in Manometric Combustion

Treatment with Ba^{++} followed by $SO_4^{=}$ was used earlier to prepare CO_2-free ammonia solutions (5), and the reaction of $Ba(OH)_2$ and Na_2SO_4 has been employed by Folch[3] to prepare NaOH solution of minimal CO_2 con-

[3] Folch, J., personal communication.

tent. The following procedure is used in place of that described by Van Slyke and Folch (p. 516 (6)) for preparation of hydrazine-containing 0.5 N NaOH solution used to absorb CO_2 in the manometric "micro" combustions (1 to 3.5 mg. of carbon) and the "submicro" combustions (1, 6, 7). The CO_2 content is about one-fourth of that of the former (6) solution. In blank analyses (p. 303 (7)) the carbonate of the solution contributes CO_2 pressures of only 1 or 2 mm. at 2 ml. volume.

A titrated solution of 0.86 N sodium hydroxide, which need not be carbonate-free, is prepared. To 2 liters of the solution are added 100 ml. of a 0.5 M barium chloride solution (122 gm. of $BaCl_2 \cdot 2H_2O$ per liter). The mixture is allowed to stand for an hour or two until most of the barium carbonate precipitate has settled. The supernatant solution is then transferred to a 2 liter Pyrex aspirator bottle, which is closed at the top with a stopper bearing a soda lime tube. The outlet at the bottom of the bottle is attached to a capillary of 2 mm. bore and about 15 cm. length, the tip of the capillary, to protect it from atmospheric CO_2, being kept under mercury which is covered with a layer of dilute acid.

After the mixture has stood overnight in the bottle, the barium carbonate has completed sedimentation and portions of the supernatant solution can be drawn as needed. To prepare a portion for use, place in a cylindrical bottle of 100 to 300 ml. capacity 2 gm. of hydrazine sulfate crystals for each 100 ml. volume. Insert the outlet tube of the aspirator bottle into the bottom of the bottle, so that the entering solution will not fall through the air, and admit alkali solution from the aspirator bottle until the receiving bottle is filled. Stopper the receiving bottle with either a rubber or a vaselined glass stopper and invert the bottle a few times till the hydrazine sulfate is dissolved and barium sulfate is precipitated. Let the stoppered bottle stand overnight for barium sulfate to settle. Then draw the supernatant solution into a cylinder protected by soda lime, as shown in Fig. 3, *D*, p. 513 of Van Slyke and Folch (6).[4]

Transfer of CO_2 from Combustion Tube to Alkali Solution in Manometric Chamber

On p. 521 of Van Slyke and Folch (6), a procedure is outlined for ejecting about two-thirds of the air from the system formed by the manometric chamber and connected combustion tube before the start of the "micro" or "submicro" combustion. With the present reagents (7) it has been

[4] If the glass cock in the outlet of this cylinder (Fig. 3, *D* (6)) is replaced by a pinchcock and rubber connecting tube, slow diffusion of atmospheric CO_2 through the rubber into the alkali solution in the bore of the rubber tube occurs. In consequence the blank on the first two or three 2 ml. portions drawn for an analysis from a cylinder that has stood for some days may be increased. To avoid error from this source, waste about 5 ml. of the alkali solution before drawing portions for the day's analyses.

found advantageous to use two successive preliminary ejections of the air, so that about 8/9 is removed from the system. After a single preliminary ejection of air enough is left so that, added to the oxygen capable of being evolved by hard boiling of the combustion mixture, enough $O_2 + N_2$ is present to retard slightly, but measurably, the subsequent absorption of CO_2 by the alkali in the manometric chamber, the effect being such that the twenty usual excursions may leave 0.5 to 1.0 per cent of the CO_2 unabsorbed. There has been no trouble from this source when two preliminary evacuations are used.

SUMMARY

The 3800 volts previously used for the determination of the specific activity of $C^{14}O_2$ in the proportional gas counter of Bernstein and Ballentine (1, 2) can be reduced to 2000 or 2100 volts by substituting for methane a mixture of 90 per cent argon and 10 per cent methane. In the method for determination of C^{14} activity by wet combustion and gas counting of the resultant mixture of $C^{14}O_2$ and $C^{12}O_2$ previously published (1), use of the lower voltage has some practical advantages.

A circuit diagram for a non-overloading amplifier, which can be used for either methane or methane-argon $C^{14}O_2$ counters, or for routine scintillation or Geiger counting, is given.

Improvements are outlined for preparing CO_2-free alkali and for transferring CO_2 during analyses.

BIBLIOGRAPHY

1. Van Slyke, D. D., Steele, R., and Plazin, J., *J. Biol. Chem.*, **192,** 769 (1951).
2. Bernstein, W., and Ballentine, R., *Rev. Sc. Instruments*, **21,** 158 (1950).
3. Katcoff, S., *Phys. Rev.*, **87,** 886 (1952).
4. Higinbotham, W. A., Brookhaven National Laboratory Report, BNL 234 (T-36) (1953).
5. Van Slyke, D. D., *J. Biol. Chem.*, **30,** 347 (1917).
6. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136,** 509 (1940).
7. Van Slyke, D. D., Plazin, J., and Weisiger, J. R., *J. Biol. Chem.*, **191,** 299 (1951).

EFFECT OF CHOLINE ADMINISTRATION ON THE OXIDATION OF FATTY ACIDS BY EXTRAHEPATIC TISSUES*

By CAMILLO ARTOM

(From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina)

(Received for publication, October 4, 1954)

It was shown previously that production of $C^{14}O_2$ from isotopic stearate or palmitate is depressed in preparations of the liver from choline-deficient rats, and that administration of choline to these animals restores the ability of the isolated tissue to oxidize the added fatty acids at a high rate (2). The present study indicates that the enhancement of fatty acid oxidation, following choline administration *in vivo*, is not specific for the liver, but occurs also in extrahepatic tissues. The most consistent increases were obtained in homogenates of kidney and heart, tissues in which fatty and necrotic changes also appear as the result of a choline deficiency.

EXPERIMENTAL

Young male albino rats (Rockland Farms; initial weight, 50 to 60 gm.) were maintained for various lengths of time on either a choline-deficient diet (Diet 26 (3), containing 5 per cent casein and 5 per cent fat, to which guanidoacetic acid, 1 per cent, was added), or a choline-supplemented diet (Diet 26, to which choline chloride, 0.4 per cent, was added).[1] A mixture of B vitamins (2) was incorporated in these diets.

In the experiments of Series I, part of the rats of a group maintained on the choline-deficient diet received choline chloride (0.25 mmole per 100 gm. of rat) in two intramuscular injections, 2 hours and 1 hour before the animals were killed. Homogenates, prepared from the tissues of these rats, were incubated simultaneously with the homogenates from the remaining rats of the same group, not injected with choline. In the experiments of Series II, the tissues of rats on the choline-deficient diet were in-

* A preliminary report has appeared (1). This work was performed under a contract (No. AT-(40-1)-1638) between the United States Atomic Energy Commission and the Bowman Gray School of Medicine. The isotopic substrates were obtained from commercial sources on allocation from the Atomic Energy Commission.

[1] In a few experiments only, guanidoacetic acid was not added to the choline-deficient diet, and, instead of Diet 26, a high fat diet (Diet 28 (4), containing 5 per cent casein and 30 per cent fat), or a low fat diet with a somewhat higher protein level (Diet 47, containing casein 8 per cent, dextrin 41, sucrose 40, Crisco 4, cod liver oil 1, Salt Mixture 2 (U. S. P. XII) 4, Ruffex 2) was used.

cubated simultaneously with those of rats which had been maintained on the choline-supplemented diet for the same length of time.

In each experiment of Series I and II, homogenates of homologous tissues from rats receiving or from those not receiving choline were brought to the same volume, but the volume varied somewhat for the various tissues and in different experiments (5 to 7 times the weight of liver, brain, and testis, 6 to 10 times the weight of kidney and heart). For each tissue, two or three experimental flasks and at least one control flask (containing the tissue homogenate, previously maintained in a boiling water bath for 10 minutes) were prepared. The incubation medium contained, in a total volume of 6 ml., 3 ml. of homogenate, 3 mg. each of penicillin G and dihydrostreptomycin, adenosine triphosphate (12 μmoles), and palmitate-1-C^{14} (1 to 2 μmoles and 1 to 2 μc.). After 3 hours of incubation in air at 37°, the $C^{14}O_2$ produced was measured. Total N was determined for the homogenates and, for the liver, total lipides and lipide P also. For further information on the preparation of the palmitate emulsion[2] and on the analytical and isotopic procedures, the reader is referred to our previous paper (2).

Results

In addition to the liver, homogenates of the following tissues were tested: kidney, heart, testis, small intestine, and skeletal muscle. The results obtained with the last two tissues are not reported, since the amounts of $C^{14}O_2$ produced under our experimental conditions were always low and mostly within the limits of the error of our measurements.

The actual data of a few typical experiments carried out with the remaining tissues are recorded in Table I. In one of these (Experiment 74), the effects on liver, kidney, and brain of injected choline, or of choline ingested and injected can be compared directly. It is apparent, however, that, as in our previous experiments on the liver (2), the amounts of $C^{14}O_2$, produced by homologous tissues under apparently similar conditions, were quite variable in different experiments. For the purpose of comparison, as well as for the sake of brevity, in Tables II and III the results of all experiments of Series I and II have been expressed as "$C^{14}O_2$ ratios;" that is, as the ratios of the $C^{14}O_2$ produced by a given tissue of the rats receiving choline to the $C^{14}O_2$ produced, in the same experiment, by the corresponding tissue of the choline-deficient animals. The ratios have been calculated from the radioactivity values per unit of tissue N. However, the same general conclusions would be reached if these values were referred to 1 gm. of moist tissue or to the whole organs of a 100 gm. rat. The differences in the neutral

[2] In most of the present experiments a small amount (0.3 mg. per ml.) of Tween 20 (Atlas Powder Company, Wilmington, Delaware) was added to the palmitate emulsion.

fat content of the livers of rats receiving or not receiving choline have also been included in Tables II and III and designated as "Change in liver fat." The *t* test of significance was applied to the means in order to decide whether these values were significantly different from 1 ($C^{14}O_2$ ratios) or from 0 (changes in liver fat).

From Table II, it appears that, when choline was injected shortly before removal of the tissues, the $C^{14}O_2$ ratios were higher than 1 in almost all of

TABLE I

$C^{14}O_2$ Production from Palmitate-1-C^{14} in Tissue Homogenates from Rats Receiving, or Not Receiving, Choline*

Experiment No.	Days on diet	Rats	Choline		Liver fat†	$C^{14}O_2$ per gm. tissue‡				
			In diet	Injected		Liver	Kidney	Heart	Brain	Testis
					mg. per gm.	*c.p.m.*	*c.p.m.*	*c.p.m.*	*c.p.m.*	*c.p.m.*
97	14	*a, b*	0	0	115	4,940	3,270	4,570		
		c, d	0	+	105	11,130	3,885	4,280		
71	24	*a, b, c*	0	0	266	1,230		500	770	1690
		d, e, f	0	+	200	3,050		940	1110	1080
68	16	*a, b, c*	0	0	97	1,950		400		1020
		d, e, f	+	+	25	3,830		1,680		980
90	17	*a, b, c*	0	0	50	9,080	4,970	3,785	3350	
		d, e, f	+	+	72	21,320	12,900	10,800	4100	
74	16	*a, b*	0	0	212	5,530	580		350	
		c, d	0	+	252	6,550	610		365	
		e, f, g	+	+	20	14,630	1,540		605	

* The number of counts per minute added to each flask as palmitate-1-C^{14} varied in the various experiments between 200,000 and 400,000.

† Total lipides (by weight) less total phospholipides (lipide P × 25).

‡ These values are the averages of determinations carried out on two or three separate flasks and have been corrected for the small values obtained on the control flask.

the experiments on liver and in a little more than half of the experiments on kidney and heart. The results of twenty experiments, in which choline was given both as a dietary supplement over a prolonged period of time and by injection shortly before death, are summarized in Table III. In five experiments (which have been grouped together arbitrarily and designated Series II-B) the $C^{14}O_2$ ratios in the liver were close to 1, and similar low values were obtained also in kidney, brain, and testis. We can only speculate on the reason, or reasons, for the discrepant results in liver and kidney. However, even in these experiments, the $C^{14}O_2$ ratios in the heart were definitely above 1. In practically all remaining experiments (Series

II-A) the liver, kidney, and heart of the rats receiving choline produced more $C^{14}O_2$ than the corresponding tissues of the choline-deficient animals.

TABLE II

Effect of Choline Administration on Oxidation of Palmitate-1-C^{14} in Tissue Homogenates; Series I

Choline injected 2 hours and 1.0 hour before death.

Experiment No.	Days on diet	No. of rats	Change in liver fat*	$C^{14}O_2$ ratios†				
				Liver	Kidney	Heart	Brain	Testis
			mg. per gm.					
65	7	6	−20	1.31	1.38	1.72		
67	11	6	−29	3.66	0.85	0.97		0.97
77	11	4	−52	1.70	1.33	1.45	1.00	
86	12	6	+34	1.88	1.50			
72	13	6	−14	1.37	1.78		0.89	
97	14	4	−10	1.86	1.29	1.08		
49	14	6	−4	4.59	0.88			
21	14‡	4	−23	0.95		1.80	0.88	
74	16	4	+40	1.24	0.98		0.95	
83	22§	4	−100	1.35	0.87	1.19		
71	24	6	−66	2.66		1.82	1.56	0.76
91	24§	6	+83	1.13			1.16	
17	26‡	3	+123	2.06				1.13
93	21§	2	+26	2.75	1.01			
16	35	4	−106	4.32	1.30	0.87		1.08
78	51	3	+254	1.81			0.94	
Means‖			+9	2.17¶	1.20**	1.36**	1.05	0.99
Standard error of means			±16.5	±0.28	±0.09	±0.13	±0.08	±0.09

* Mg. of fat in the livers of rats receiving choline less mg. of fat in the liver of rats not receiving choline. Liver fat = total lipides (by weight) less total phospholipides (lipide P × 25).

† Ratio of $C^{14}O_2$ produced by the tissues of rats not receiving choline to $C^{14}O_2$ produced by the tissues of rats receiving choline. $C^{14}O_2$ expressed as counts per minute per mg. of tissue N.

‡ Diet 47.

§ Diet 28.

‖ Unmarked values are not significantly different from 0 (change in liver fat), or from 1 ($C^{14}O_2$ ratios) ($P > 0.05$).

¶ Values significantly different from 1 ($P < 0.01$).

** Values significantly different from 1 ($P < 0.05$).

In both Series I and II, $C^{14}O_2$ ratios greater than 1 were observed occasionally in brain, but never in testis.

As expected, values for the "Change in liver fat," were quite variable

TABLE III

Effect of Choline Administration on Oxidation of Palmitate-1-C^{14} in Tissue Homogenates; Series II

Choline added to the diet and injected 2.0 hours and 1.0 hour before death.

Series No.	Experiment No.	Days on diet	No. of rats	Change in liver fat*	$C^{14}O_2$ ratios†				
					Liver	Kidney	Heart	Brain	Testis
				mg. per gm.					
II-A	85	9	6	−41	1.14	1.74	1.23		
	59	10	8	−132	1.62	1.64	1.47		
	53	12	8	−86	2.10	1.93	1.25		
	72	13	6	−164	2.54			0.99	
	68	16	6	−72	1.71		3.34		0.90
	74	16	5	−192	2.51	2.41		1.37	
	90	17	6	+22	2.49	2.64	2.63	1.03	
	10	18	4	−11	2.46	1.57	3.70		
	70	19	8	−131	1.27	1.85			0.87
	5	21	2	−53	3.20	1.70			
	23	22‡	4	−27	2.46	1.37	1.30	2.28	
	99	24	6	−88	1.19	2.70	1.02	1.02	
	25	29‡	4	−11	2.80	1.35	1.88	1.40	
	12	30	2	+2	2.46	1.53			1.06
	84	30	4	−146	1.48	1.26			
Means§				−75‖	2.10¶	1.82¶	1.98**	1.35	0.94
Standard error of means				±17	±0.15	±0.13	±0.33	±0.20	±0.06
II-B	79	11	6	−47	1.02	0.86	1.48	0.86	
	75	13	8	−72	0.97	0.91	1.13	1.22	0.97
	82	14††	6	−111	0.94	0.97	1.23	0.86	
	4	17	4	−57	1.00	1.08	1.44		1.08
	76	18	6	−153	0.99	1.01		1.16	
Means§				−88‡‡	0.99	0.97	1.32**	1.03	1.03
Standard error of means				±20	±0.01	±0.04	±0.08	±0.10	±0.06

* See Table II.
† See Table II.
‡ Diet 47.
§ Unmarked values are not significantly different from 1 ($P > 0.05$).
‖ Significantly different from 0 ($P < 0.01$).
¶ Significantly different from 1 ($P < 0.01$).
** Significantly different from 1 ($P < 0.05$).
†† Diet 28.
‡‡ Significantly different from 0 ($P < 0.05$).

in the experiments of Series I. On the other hand, the lipotropic effect of the choline-supplemented diet was apparent in almost all of the experiments of Series II (including those of Series II-B, in which the rate of fatty acid oxidation in the isolated liver of rats receiving choline was not increased).[3]

DISCUSSION

Since choline administered *in vivo* enhanced the oxidation of fatty acids in the isolated liver, whereas addition *in vitro* of choline or of simple choline derivatives was ineffective, we postulated that such choline effect was due to some complex substance (or substances) formed from choline *in vivo*, but not *in vitro*, at least under the conditions of our experiments (2). Results obtained in a study of the oxidation of octanoate in choline-deficient livers lead to a similar interpretation (5). It appears now that the active factor (or factors) is formed also in some extrahepatic tissues, such as kidney and heart, although presumably at a rate slower than in the liver. This is suggested by the finding that in the shorter periods after choline was given (Series I) $C^{14}O_2$ production was increased in the liver much more consistently than in kidney and heart, whereas in the experiments in which choline was administered over prolonged periods the enhancement of fatty acid oxidation in the liver was accompanied almost always by a similar increase in kidney and heart (Series II-A). Thus, if the postulated active factors were choline-containing phospholipides, our results would be in line with previous indications that the rate of phospholipide synthesis, as measured by the incorporation of isotopic phosphate (6), choline (7), or fatty acid (8), is definitely lower in kidney and heart than in liver.

Aside from such speculations, the present findings are of interest in view of other reports in the literature. Indeed, the hemorrhagic kidney syndrome of young rats fed low choline diets has been known for years (9), and in this condition early deposition of fat in the cells of the proximal convoluted tubules has been noted (10). Necrotic and degenerative lesions have been described in the myocardium and in the coronary and aortic walls of rats fed various types of choline-deficient diets (11–13); these changes are preceded invariably by an accumulation of fat, which can be prevented by adding choline to the diet (14).

The excellent technical assistance of Miss B. Blue and Mrs. E. Hamrick Brady is gratefully acknowledged.

[3] A tentative explanation for the lack of correlation between the effects of choline administration on the fat content of the liver and on the rate of fatty oxidation in the isolated tissue has been suggested previously (2).

SUMMARY

Homogenates of the liver, kidney, heart, brain, and testis from rats maintained on low protein diets were incubated in air with palmitate-1-C^{14}, and the amounts of $C^{14}O_2$ produced during the incubation were determined.

Following the injection of choline shortly before the animals were killed, higher $C^{14}O_2$ values were obtained in liver and often in kidney and heart also. When choline was given both as a dietary supplement and by injection terminally, $C^{14}O_2$ production was generally enhanced in all three tissues to about the same extent.

It is suggested that the formation *in vivo* of the active factors responsible for the increased rate of fatty acid oxidation *in vitro* occurs also in extrahepatic tissues, but that such factors are formed in kidney and heart at a rate which is often slower than in liver.

Increases in $C^{14}O_2$ production after choline administration were observed in brain occasionally, but never in testis.

BIBLIOGRAPHY

1. Artom, C., *Federation Proc.*, **13,** 176 (1954).
2. Artom, C., *J. Biol. Chem.*, **205,** 101 (1953).
3. Artom, C., and Cornatzer, W. E., *J. Biol. Chem.*, **171,** 779 (1947).
4. Cornatzer, W. E., and Artom, C., *J. Biol. Chem.*, **178,** 775 (1949).
5. Lévy, M., and Legrand, J., *Arch. sc. physiol.*, **7,** 63 (1953).
6. Artom, C., Perrier, C., Santangelo, M., Sarzana, G., and Segrè, E., *Nature*, **139,** 846 (1937).
7. Stetten, D., Jr., *J. Biol. Chem.*, **140,** 143 (1941).
8. Goldman, D. S., Chaikoff, I. L., Reinhardt, W. O., Entenman, C., and Dauben, W. G., *J. Biol. Chem.*, **184,** 727 (1950).
9. Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, **131,** 567 (1939).
10. Hartroft, W. S., and Best, C. H., *Science*, **105,** 315 (1947).
11. Kesten, H. D., Salcedo, J., Jr., and Stetten, D., Jr., *J. Nutr.*, **29,** 171 (1945).
12. Hartroft, W. S., Ridout, J. H., Sellers, E. A., and Best, C. H., *Proc. Soc. Exp. Biol. and Med.*, **81,** 384 (1952).
13. Wilgram, G. F., and Hartroft, W. S., XIXth International Physiological Congress, Abstracts Communications, Montreal, 890 (1953).
14. Wilgram, G. F., Hartroft, W. S., and Best, C. H., *Science*, **119,** 842 (1954); *Brit. Med. J.*, **2,** 1 (1954).

STUDIES ON THE MECHANISM OF CARBAMYLASPARTIC ACID SYNTHESIS*

By J. M. LOWENSTEIN and PHILIP P. COHEN

(From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin)

(Received for publication, October 11, 1954)

Evidence that carbamylaspartic acid is a precursor of orotic acid in both bacteria and mammals has been presented by several workers (1–4). The literature on the fate of orotic acid, a precursor of two or more pyrimidine nucleotides, has been reviewed by Reichard and Lagerkvist (1). More recently, Cooper and Wilson (4) have confirmed the conversion of orotic acid to nucleic acid uracil and cytosine, and Kornberg *et al.* (5, 6) have demonstrated several of the individual steps of this conversion. An experiment *in vivo* with citrulline labeled with C^{14} and N^{15} in the carbamyl group led Cooper and Wilson (4) to conclude that the urea cycle was not involved in the formation of CA[1] in the rat. Similar experiments were performed by Schulman and Badger (7), who found that C^{14}-carbamyl-labeled citrulline gave rise to labeled nucleic acid pyrimidines in the pigeon but not in the rat. The latter findings indicate that the pathway of CA synthesis in mammals may differ from that in birds. The results of both of these groups are in apparent contradiction to those of Smith and Stetten (8), who found that C^{14}-carbamyl-labeled citrulline gave rise to labeled orotic acid with rat but not with pigeon liver slices.

In preliminary experiments we obtained evidence that extracts of whole rat liver acetone powder catalyze the formation of CA when incubated with aspartate, acetyl glutamate, CO_2, ammonia, ATP, and Mg ions.[2] Very recently Reichard (9) showed that rat liver mitochondrial preparations form CA from aspartate, CO_2, and ammonia in the presence of glutamic acid or carbamylglutamic acid, and ATP or a source of high energy phosphate. The pathway

$$CO_2 + \text{ammonia} + \text{ATP} + \text{acetylglutamic acid}^3 \longrightarrow \text{intermediate (10, 11)} \xrightarrow{\text{ornithine}}$$

$$\text{citrulline} \xrightarrow{\text{aspartic acid}} \text{argininosuccinic acid (12, 13)} \longrightarrow \text{CA} + \text{ornithine}$$

* This investigation was supported in part by grants from the National Institutes of Health, United States Public Health Service, and the Wisconsin Alumni Research Foundation.

[1] The abbreviations used in this paper are CA, carbamylaspartic acid, and ATP, adenosinetriphosphate.

[2] Unpublished observations.

[3] Or one of the other precursors of the intermediate (10, 11).

XUM

appeared an attractive possibility, but we could find no evidence of its occurrence in rat liver.[2]

Another possibility is that CA might be formed by the reaction: intermediate + aspartic acid → CA. The present paper presents evidence for this reaction. A preliminary report of this finding has been published (14).

EXPERIMENTAL

Preparation of C^{14}-Labeled Intermediate—A solution of labeled intermediate was prepared as follows (10, 11) (the quantities are in micromoles). Acetyl glutamate 400, $C^{14}O_2$ 280, NH_4Cl 800, $MgSO_4$ 400, 0.1 M phosphate buffer, pH 7.4, 400, ATP 320, and 40 per cent ethanol-precipitated fraction[4] of an extract of washed rat liver residue acetone powder (15), 150 mg., were incubated in a volume of 22 ml. at 37.5° for 30 minutes. The solution was deproteinized with 5 ml. of 1 M $HClO_4$ under ice-cold conditions. After standing for 4 minutes, the protein precipitate was removed by centrifugation at 2000 × *g* for 4 minutes. The solution containing the intermediate was decanted into a round bottomed flask and placed in an ice bath. The flask was evacuated for 30 minutes in order to remove unchanged $C^{14}O_2$. This was facilitated by admitting CO_2 gas into the solution through a capillary tube. The solution was neutralized to pH 7.2 with 6 ml. of 1 M $NaHCO_3$ and assayed for radioactivity and intermediate (10). The solution was then frozen. We have found that solutions of the intermediate may be kept in the frozen state for as long as 2 weeks with little loss in activity.

The experiments reported in this paper were performed with two solutions of the intermediate prepared in the above manner. Preparation 1 contained 0.936 μmole of intermediate per ml., specific activity 3.49×10^5 c.p.m. per μmole; total yield 27.5 ml. Preparation 2 contained 0.800 μmole of intermediate per ml., specific activity 1.69×10^5 c.p.m. per μmole; total yield 33.0 ml. (A larger volume (26 ml.) was used for the incubation of Preparation 2.)

The use of 6000 μmoles of $NaHCO_3$ to neutralize the deproteinized reaction mixture considerably diluted the specific activity of any unchanged $C^{14}O_2$ which might still have been present in the solution of the intermediate. Thus even if no $C^{14}O_2$ had reacted to form the intermediate and if none had been removed by the acidification and evacuation, the specific activity of the $C^{14}O_2$ would have been diluted 22-fold.

Formation of Carbamylaspartic Acid—6.8 ml. of the solution of intermediate (Preparation 1, 6.36 μmoles), 1 ml. of aspartate (100 μmoles), and 1 ml.

[4] R. O. Marshall and S. Grisolia found that this fraction was satisfactory for the preparation of the intermediate (personal communication).

of enzyme solution (an aqueous extract of lyophilized rat liver supernatant solution (16) containing 104 mg. of protein per ml.) were incubated at pH 7.2 to 7.4, 37.5°, for 10 minutes. Tubes with water in place of aspartate served as controls. The reactions were stopped and the solutions deproteinized by the addition of 3 ml. of ice-cold 1 M $HClO_4$. The tubes were placed in an ice bath for 5 minutes and were then centrifuged at 2000 × *g* for 5 minutes. The supernatant solutions were decanted and neutralized to pH 8 with KOH. The mixtures were chilled to near freezing, and were

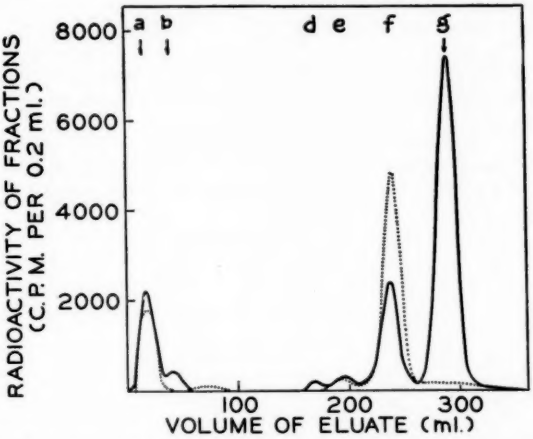


FIG. 1. The formation of CA by lyophilized rat liver supernatant solution. Solid line, aspartate plus labeled intermediate; broken line, aspartate replaced by water. The arrows indicate the positions of the elution peaks of authentic samples of (*a*), citrulline (arginine, urea); (*b*), argininosuccinate; (*g*), carbamyl aspartate (DL form). The identity of Peaks *d*, *e*, and *f* has not yet been established.

centrifuged at 2000 × *g* for 5 minutes to remove precipitated $KClO_4$. The supernatant solutions were frozen until used for analysis.

The solutions were subjected to anion exchange chromatography by gradient elution technique (17, 18). The resin employed was Dowex 2 × 10, 200 to 400 mesh, formate form, in columns 10.5 cm. long and 1.1 cm. in diameter. The reservoir of the gradient elution apparatus contained 0.6 M ammonium formate-formic acid buffer, pH 4.0, and the mixing vessel 500 ml. of water. Fractions of about 5 ml. were collected and 0.2 ml. of each fraction was assayed for radioactivity. Several radioactive compounds were detected in this manner (Fig. 1). Elution peaks presumed to contain CA were identified by comparison with the position of the elution peak of authentic samples of CA (DL form). Individual fractions con-

tained presumed CA in amounts insufficient for determination by the colorimetric method. The fractions in question were pooled and freeze-dried, and the buffer salt was removed by sublimation *in vacuo* under a radiant lamp. The residue was dissolved in 5 ml. of water, and the resulting solution (hereafter called Solution A) was assayed for radioactivity, and for CA by the colorimetric method of Koritz and Cohen (19) with authentic CA as a standard. Solution A was found to contain 5.24×10^5 c.p.m. per ml., and 0.161 μmole per ml. expressed as CA. Hence the specific activity of the presumed CA formed during the incubation was

TABLE I

Identification of Radioactive Substance in Solution A by Chromatography of 1.48×10^4 C.p.m. of Solution A with 10 μmoles of Authentic CA (DL *Form*)

Fraction No.	C.p.m. per ml.	μmole CA per ml.	C.p.m. per μmole CA
52	0		
53	10		
54	124	0.070	177
55	448	0.265	169
56	847	0.496	171
57	714	0.368	194
58	419	0.233	180
59	217	0.121	179
60	115	0.067	172
61	50		
62	15		
63	0		

3.25×10^5 c.p.m. per μmole. This compares with a specific activity of 3.49×10^5 c.p.m. per μmole for the intermediate.

The remainder of Solution A was used to identify the presumed CA as described below. (*a*) To a portion of Solution A containing 1.48×10^4 c.p.m. were added 10 μmoles of CA (DL form). The solution was chromatographed, and the fractions representing the elution peak of the added CA were assayed colorimetrically and also for radioactivity. The specific activity of CA was constant within the limits of experimental error (Table I). (*b*) To a portion of Solution A containing 3.84×10^4 c.p.m. were added 500 mg. of CA (DL form). The carrier compound was dissolved and allowed to recrystallize. After filtration, the crystals were divided into two equal portions. One portion was recrystallized from hot water four times, and the specific activity and melting point of the compound were determined after each recrystallization. The other portion was converted into hydantoin-5-acetic acid by dissolving it in 2 ml. of 5 N HCl, refluxing

for 1 hour, and evaporating the solution almost to dryness (20). The residue was recrystallized from hot water twice, and the melting point and specific activity of the compound were determined after each recrystallization. The results are shown in Table II.

An attempt was made to remove the compounds which give rise to Peaks *a*, *b*, *d*, *e*, and *f* (Fig. 1) by dialyzing the lyophilized rat liver supernatant solution. 2.6 gm. of this preparation were dissolved in 26 ml. of water and dialyzed against 14 liters of ice-cold distilled water for 11 hours. The precipitated protein was removed by centrifugation and the supernatant solution was lyophilized. The resulting preparation was completely soluble in water and was used in the experiments described below. Com-

TABLE II

Isotope Dilution Experiments with Solution A

Solution A, 3.84×10^4 c.p.m.; carrier, 500 mg. of CA (DL form). After the first recrystallization, one-half of the product was converted to hydantoin-5-acetic acid.

	Recrystallization No.	M.p.	Specific activity, c.p.m. per mmole
		°C.*	
Carbamylaspartic acid	1	174	14,000
	2	174	13,370
	3	174	13,710
	4	174	13,230
Hydantoin-5-acetic acid	1	212	13,490
	2	212	13,490

* Uncorrected.

pared on a protein basis with the undialyzed preparation, it synthesized 52 per cent more CA, 37 per cent more citrulline, 42 per cent less argininosuccinate, and 30 per cent less of Peak *f* with aspartate as substrate.

Influence of Ornithine, Citrulline, and Argininosuccinate on Formation of Carbamylaspartic Acid—1.0 ml. of the solution of intermediate (Preparation 2, 0.800 μmole), 20 mg. of dialyzed enzyme preparation, and various combinations of aspartate, ornithine, citrulline (20 μmoles each), and argininosuccinate (10 μmoles) were incubated in a final volume of 1.4 ml., pH 7.2 to 7.4, at 37.5° for 15 minutes. The reaction was stopped and the solutions were subjected to ion exchange chromatography, as described in the previous section. After determining the elution pattern in the manner shown in Fig. 1, the total radioactivity of each peak was determined under standardized conditions. As a check, fractions corresponding to the peaks were freeze-dried and the buffer salts removed by sublimation *in vacuo*. The residues were dissolved in water and samples were chromatographed

on paper with phenol saturated with citrate-phosphate buffer (21) as the descending solvent. The samples were run next to and superimposed with authentic amino acids, urea, and CA. The spots were detected by spraying the dried paper sheets with ninhydrin or *p*-dimethylaminobenzaldehyde hydrochloride, and, where pertinent, by cutting the sheets into squares and measuring their radioactivity in a gas flow counter. R_F values in a typical run of 28 cm. were CA 0.11, arginine 0.57 (drawn out spot), citrulline 0.62 (sharp spot), urea 0.73. Argininosuccinate usually gave two spots, R_F 0.18 (sharp spot) and R_F 0.48 (drawn out spot), when taken through the isolation procedure described above. The drawn out spot presumably represented argininosuccinic anhydride, an isolation artifact (13). The results are shown in Table III.

TABLE III

Influence of Ornithine, Citrulline, and Argininosuccinate on Synthesis of CA

Each tube contained, where indicated, amino acids 20 μmoles (except argininosuccinate 10 μmoles), and enzyme (dialyzed, lyophilized rat liver supernatant fluid) 20 mg. Intermediate, 0.800 μmole, 1.352×10^5 c.p.m., was added to each tube at the start of the incubation. Final volume 1.4 ml., pH 7.2 to 7.4, 37.5°, 15 minutes. The reaction was stopped with 1 ml. of 1 M $HClO_4$. The residue was removed by centrifugation. The supernatant fluid was neutralized to pH 7 to 8 with KOH and centrifuged to remove $KClO_4$. The supernatant fluid was chromatographed on Dowex 2 formate.

Tube	Amino acids	Total c.p.m. of elution peaks* ($\times 10^{-3}$)			
		Citrulline	Arginino-succinate	Peak *f* (unidentified)	CA
A†	None	1.5	0	5.0	0
B	"	14.5	0	7.9	0
C	Aspartate	11.3	3.2	5.8	88.7
D	Ornithine	126.5	0	2.3	0
E	Aspartate, ornithine	85.1	22.2	2.7	20.8
F	Citrulline	15.9	0	3.9	0
G	Aspartate, citrulline	17.3	0.7	2.8	80.4
H	Argininosuccinate	87.6	0	5.0	0
J	Aspartate, argininosuccinate	72.9	6.3	2.3	36.7

* Identified (1) by the volume of eluate at which the peak was eluted, (2) by paper chromatography of pooled fractions of peaks after freeze-drying and removal of buffer salts by sublimation, and (3) (in the case of CA only) by isotope dilution.

† No enzyme added.

DISCUSSION

Formation of CA—The data presented in this paper provide evidence that the carbamyl and aspartate moieties of CA originate from the inter-

mediate and aspartate respectively. The conclusion that the intermediate is involved is based on specific activity data which preclude other, independent CO_2 fixation mechanisms.

Effect of Ornithine, Citrulline, and Argininosuccinate on Synthesis of CA from Aspartate and Intermediate—When ornithine is added to the system containing aspartate, there is a large decrease in the radioactivity incorporated into CA (Table III, Tubes C and E). The simplest explanation of the effect of ornithine is that it and aspartate act as competitive substrates for the transcarbamylation reaction. The decrease in the radioactivity incorporated into CA, on adding citrulline to the system containing aspartate, is small, and is of the same order as the increase in radioactivity incorporated into citrulline (Tubes C and G). This finding suggests that citrulline is not an intermediate in the formation of CA in this system. The effect of argininosuccinate on the incorporation of radioactivity into CA is not only similar to the effect of ornithine but is probably due to ornithine, since the amount of radioactive citrulline formed in the system containing argininosuccinate alone is of the same order (actually somewhat greater) as the amount formed in the system containing argininosuccinate and aspartate (Tubes H and J). The ornithine in this case is probably produced enzymatically from argininosuccinate, through the subsequent steps of the urea cycle.

Rôle of Argininosuccinate—There is a sharp reduction in the radioactivity incorporated into argininosuccinate when ornithine is replaced by citrulline (Tubes E and G). One explanation of this effect is that the specific activity of the carbamyl group is lowered by the citrulline pool, and that the lower radioactivity of argininosuccinate in Tube G is a reflection of this lower specific activity rather than the amount of argininosuccinate synthesized. If this were the case, argininosuccinate could not be an intermediate in the formation of CA (see Tubes C and G). An alternative explanation is that the formation of argininosuccinate from aspartate requires ATP only when citrulline is involved, and not when it is formed from the intermediate and ornithine. Neither explanation accounts for the reduction in the radioactivity incorporated into CA in the system containing aspartate and ornithine, unless ornithine, citrulline, and argininosuccinate are not required for and are not intermediates in the formation of CA.

The possibility that the intermediate acts as a more general donor of the carbamyl group and the purification of the enzymes involved in CA synthesis are under investigation.

We wish to thank Dr. Sarah Ratner for a most generous gift of Ba argininosuccinate, and Mr. Richard O. Marshall for advice and help in the preparation of ethanol fractions of washed rat liver residues.

SUMMARY

Evidence has been presented that the intermediate involved in the synthesis of citrulline from ornithine also reacts with aspartate in the presence of soluble liver preparations to form carbamyl aspartate.

The influence of ornithine, citrulline, and argininosuccinate on the latter system has been investigated. The interpretation of the data presented is that these amino acids are not directly involved in the synthesis of carbamyl aspartate.

BIBLIOGRAPHY

1. Reichard, P., and Lagerkvist, V., *Acta chem. Scand.*, **7**, 1207 (1953).
2. Lieberman, I., and Kornberg, A., *J. Biol. Chem.*, **207**, 911 (1954).
3. Lieberman, I., and Kornberg, A., *Biochim. et. biophys. acta*, **12**, 223 (1954).
4. Cooper, C., and Wilson, D. W., *Federation Proc.*, **13**, 194 (1954).
5. Lieberman, I., Kornberg, A., Simms, E. S., and Kornberg, S. R., *Federation Proc.*, **13**, 252 (1954).
6. Kornberg, A., Lieberman, I., and Simms, E. S., *J. Am. Chem. Soc.*, **76**, 2027 (1954).
7. Schulman, M. P., and Badger, S. J., *Federation Proc.*, **13**, 292 (1954).
8. Smith, L. H., Jr., and Stetten, D., Jr., *J. Am. Chem. Soc.*, **76**, 3864 (1954).
9. Reichard, P., *Acta chem. Scand.*, **8**, 795 (1954).
10. Grisolia, S., and Cohen, P. P., *J. Biol. Chem.*, **198**, 561 (1952).
11. Grisolia, S., and Cohen, P. P., *J. Biol. Chem.*, **204**, 753 (1953).
12. Walker, J. B., and Myers, J., *J. Biol. Chem.*, **203**, 143 (1953).
13. Ratner, S., Petrack, B., and Rochovansky, O., *J. Biol. Chem.*, **204**, 95 (1953).
14. Lowenstein, J. M., and Cohen, P. P., *J. Am. Chem. Soc.*, **76**, 5571 (1954).
15. Grisolia, S., and Cohen, P. P., *J. Biol. Chem.*, **191**, 189 (1951).
16. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **172**, 405 (1948).
17. Lakshmanan, T. K., and Lieberman, S., *Arch. Biochem. and Biophys.*, **45**, 235 (1953).
18. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209**, 23 (1954).
19. Koritz, S. B., and Cohen, P. P., *J. Biol. Chem.*, **209**, 145 (1954).
20. Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, **69**, 1382 (1947).
21. Berry, H. K., Sutton, H. E., Cain, L., and Berry, J. S., *Univ. Texas Pub., No. 5109*, 22 (1951).

THE INCORPORATION OF FORMATE-C^{14} INTO CITROVORUM FACTOR*

By SIGMUND F. ZAKRZEWSKI and CHARLES A. NICHOL†

(From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut)

(Received for publication, July 28, 1954)

Jukes (1) and Broquist *et al.* (2) reported that the addition of formate or serine to incubating cells of an A-Methopterin-resistant strain of *Streptococcus faecalis* increased the amount of *citrovorum* factor (CF, folinic acid) which was formed from pteroylglutamic acid (PGA). This finding suggested that "formate-carbon" was incorporated into the structure of CF. Under appropriate conditions an excellent yield of CF is obtainable (3), so that an isolation of CF from bacterial suspensions incubated with PGA is feasible. It is the purpose of this paper to report the preparation of labeled CF which was derived enzymatically from synthetic pteroylglutamic acid and formate-C^{14}.

Methods and Results

Resting Cell Suspension—Cells from a 16 hour culture of *S. faecalis* A (4), grown in a medium (5) supplemented with PGA (2 mμgm. per ml.), were spun down and washed once with cold saline. The cells (10 mg. per ml., wet weight) were incubated for 4 hours at 37° under an atmosphere of nitrogen in phosphate buffer (0.1 M, pH 6.5) containing PGA (200 γ per ml.), sodium formate-C^{14} (0.002 M; 1.54×10^8 c.p.m. per mmole), sodium ascorbate (0.01 M), and glucose (0.02 M). The incubation mixture was heated at 120° for 30 minutes. The cells were removed by centrifugation and the clear supernatant fluid was assayed for its activity for *Leuconostoc citrovorum* (8081) (6) and for *S. faecalis* (8043) (5). The total folic acid-like activity of the solution was 202 γ per ml., of which 82 γ per ml. were measurable as CF. Accordingly, the yield of CF in 900 ml. of the incubation mixture was 73.8 mg. This measurement is expressed in terms of the naturally occurring CF, which is twice as potent as the synthetic material that was used as a reference standard for this assay (7, 8).

* This investigation was supported in part by a research grant from the National Institutes of Health, United States Public Health Service, and in part by a departmental grant from the Lederle Laboratories Division of the American Cyanamid Company. This paper was presented at the annual meeting of the American Institute of Nutrition at Atlantic City, April, 1954.

† Scholar in Cancer Research of the American Cancer Society.

Paper Chromatography—Samples of the incubation mixture were placed on strips of Whatman No. 1 filter paper, which were developed by descending phosphate buffer (0.1 M, pH 7.0). The distribution of the compounds having folic acid-like activity was determined by placing the dried strips on the surface of solid medium which was seeded with *S. faecalis* (9, 10). Four zones of growth were observed following incubation of the assay plates at 37° for at least 6 hours (R_F 0.36 (I), 0.59 (II), 0.74 (III), and 0.86 (IV)). Two zones of growth were observed when duplicate strips were placed on medium seeded with *L. citrovorum* (R_F 0.74 and 0.86). The R_F values and absorption spectra of Compounds I and III correspond to those of synthetic PGA and CF,[1] respectively (11, 12). Identification of Compounds II and IV has been impeded by the lability of II in neutral solutions and the occurrence of IV in only very small amounts. Preliminary studies of II indicate that it is readily degraded to 10-formyl-PGA, which has been identified by its absorption spectrum and by paper chromatography as a product occurring in fractions eluted from Dowex 1 chloride.[2]

Isolation of Citrovorum Factor—The incubation mixture was chilled in an ice bath, adjusted to pH 4.5, and treated twice with charcoal (Darco G-60). The filtrate contained only trace amounts of folic acid. Elution of 88 per cent of the original folic acid-like activity was accomplished by stirring the charcoal with C_2H_5OH:NH_4OH:H_2O (4:1:3) at 40°, filtering, and repeating this treatment. The combined eluates were concentrated to a small volume under reduced pressure. An ion exchange column (Dowex 1-acetate, 4 per cent cross-linked, 200 to 400 mesh) was used to separate CF from unaltered PGA. A portion of the charcoal eluate which contained about 10 mg. of CF activity was adjusted to pH 8 and was placed on the resin. The column was washed with distilled water, and fractions eluted by increasing concentrations of acetate buffer (pH 4.7) were collected. The folic acid-like activity, CF activity, and radioactivity of the fractions were determined (Fig. 1). Because the high concentration of salt interfered with the counting procedures, the comparative radioactivity of the fractions was determined following elution of the active material from charcoal. Aliquots (1.0 ml.) of the fractions were shaken with approximately 5 mg. of Darco G-60, centrifuged, and the charcoal treated twice with 1 ml. portions of the elution mixture. The eluates were dried in planchets and were counted directly with an end window Geiger tube (Tracerlab TGC-2).

[1] The R_F of PGA has been observed to vary when different amounts are placed on the paper strips. With 0.05 γ per 1 inch strip, the R_F was 0.36 (bioautogram). With 250 γ per 1 inch strip, the R_F was 0.46 (ultraviolet-absorbing zone). Such variation has not been observed when similar amounts of CF were used.

[2] Zakrzewski, S. F., and Nichol, C. A., unpublished data.

Two peaks of microbial activity, as measured by *S. faecalis*, were obtained. The first peak contained CF, whereas the second peak was without activity for *L. citrovorum*. A peak of radioactivity corresponded to the fractions containing CF. These latter fractions were combined. The active material was adsorbed on Darco G-60 and eluted with ethanol-ammonia-water. The remainder of the incubation mixture was treated in the same manner.

The charcoal eluates were concentrated to a small volume. Calcium chloride was added and the solution was neutralized. CF was precipitated from ethanol as the calcium salt at −10°. The precipitate was dissolved

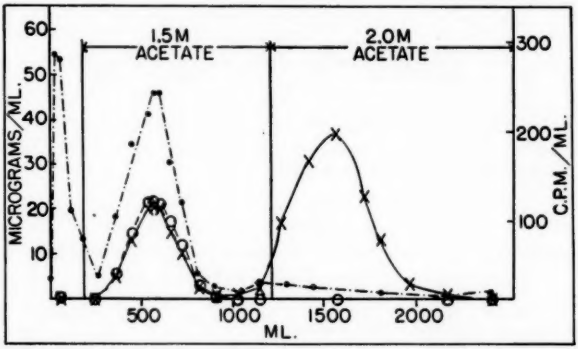


FIG. 1. Comparison of microbial activity and radioactivity of fractions eluted from a Dowex 1 column. ×, activity for *S. faecalis;* ○, activity for *L. citrovorum;* and ●, radioactivity measured as described in the text.

in minimal amounts of water (0.5 to 1.0 ml.) and reprecipitated twice from ethanol.

Paper chromatography was used for the final purification of CF. This procedure separated CF from a microbially inactive fluorescing substance (R_F 0.80) which was present in the precipitate from ethanol. Strips or sheets of Whatman No. 1 filter paper were developed by descending solvent (0.1 M phosphate buffer, pH 7.0). The ultraviolet-absorbing zone at R_F 0.74 was eluted by distilled water. The over-all yield of CF was 5 per cent of the activity of the crude incubation product.

Identification of Citrovorum Factor—The ultraviolet absorption spectrum (0.1 N NaOH) of the compound which was eluted from the paper closely resembled that of synthetic CF (Fig. 2) (11, 12).

The microbial potency of the isolated CF was twice that of synthetic CF (leucovorin) in promoting the growth of *S. faecalis* and of *L. citrovorum*, based upon equivalent ultraviolet absorption of the solutions (Table I).

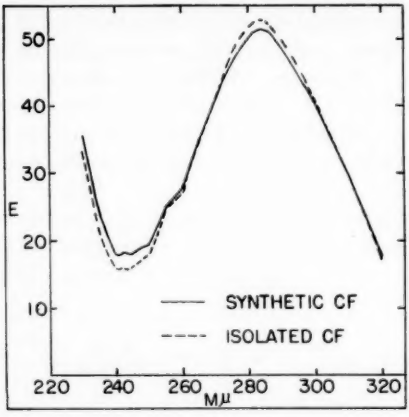


FIG. 2. Comparison of the ultraviolet absorption spectra of synthetic and isolated *citrovorum* factor. The solutions used for the measurement of *E* contained 10 mg. of CF per liter dissolved in 0.1 N sodium hydroxide.

TABLE I

Microbial Potency and Stability of Synthetic and Isolated Citrovorum Factor

Compound	Concentration	Microbial assay			
		S. faecalis (8043)		*L. citrovorum* (8081)	
	γ per ml.	*γ per ml.*	*per cent activity*	*γ per ml.*	*per cent activity*
Pteroylglutamic acid	20.0*	†	100		
Ca leucovorin	20.0*	10.1	50.5	†	50
Ba "	26.8*	12.8	47.8		
Ca "	20.0*	9.82	49.1	10.1	50.5
" "	10.0*‡	5.10	51.0	5.02	50.2
Isolated CF	8.81‡	8.62	97.8	8.96	102
Ca leucovorin					
Aqueous		11.3		11.0	
0.1 N NaOH§		10.6		9.68	
0.1 " HCl§		11.5		0.35	
Isolated CF					
Aqueous		10.6		9.98	
0.1 N NaOH§		11.8		9.77	
0.1 " HCl§		9.26		0	

* Weight of the free anhydrous acid.

† Reference standard.

‡ Weight of the free anhydrous acid correlated with ultraviolet absorption spectrum.

§ 20 hours at room temperature.

This observation confirms the finding by Keresztesy and Silverman (7) that naturally occurring CF had twice the activity of synthetic CF, a circumstance to be accounted for by the occurrence of a biologically inactive diastereoisomer in the synthetic product (8).

Examination of paper chromatograms of the product (100 γ per 1 inch strip) under ultraviolet light indicated the presence of a single zone of absorption at the same R_F as synthetic CF (Fig. 3). Although fluorescing compounds are readily detected by this procedure, none was visible.

Bioautograms of the product (1 γ per 1 inch strip) showed a single zone with activity for *S. faecalis*. Duplicate bioautograms with *L. citrovorum*

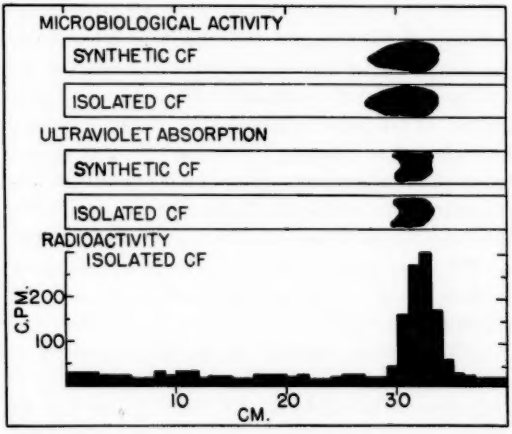


FIG. 3. Chromatograms of synthetic and isolated *citrovorum* factor

showed that this zone corresponded to that of synthetic CF (Fig. 3). This technique would have indicated the presence of folic acid-active compounds of other R_F values if such substances were present in a concentration exceeding 0.5 per cent of the CF activity (10).

Formyl-C^{14}-Citrovorum Factor—A single zone of radioactivity was observed when 1 cm. sections of a paper chromatogram (100 γ of isolated CF per $\frac{1}{2}$ inch strip) were counted directly with an end window Geiger tube. This zone corresponded with the zone of CF activity observed on the bioautograms and to the zone of absorption which was visible under ultraviolet light (Fig. 3).

The specific activity of the isolated CF, counted directly with a windowless flow counter (Tracerlab SC-16), was 96,000 c.p.m. per μmole. The enzymatic formation of CF by this system apparently involved very little

dilution of the formate-C^{14}, since the specific activity of the CF was 0.6 of the specific activity of the formate which was used.

Treatment of either the isolated CF or leucovorin with 0.1 N NaOH did not result in appreciable loss of microbial activity (Table I), but exposure to 0.1 N HCl destroyed the activity of each for *L. citrovorum* but not for *S. faecalis* (Table I). These properties are in agreement with those reported by Keresztesy and Silverman (13) for preparation of *citrovorum* factor which were derived from liver and with the original observation by Bardos *et al.* (14) that mild acid hydrolysis inactivated natural folinic acid while activity for *S. faecalis* was retained. The chemistry of the acid degradation products of CF has been studied in detail (12, 15, 16).

Treatment of the labeled CF with acid (2 N HCl for 24 hours at room temperature), followed by alkaline treatment under aerobic conditions, resulted in the formation of an acid-volatile radioactive product which accounted for 25 per cent of the original radioactivity. The acid degradation and hydrolysis of CF were not quantitative since several compounds other than PGA which had activity for *S. faecalis* were detected on paper chromatograms of the hydrolysate. The small amount of labeled CF was not adequate to permit the isolation of non-radioactive PGA which should result from this treatment if the radioactivity were located solely in a formyl group. CF (5-formyl-5,6,7,8-tetrahydropteroylglutamic acid) is remarkably stable to alkali, but upon exposure to acid it is converted to an imidazolinium compound which has a carbon bridge linking the N-5 and N-10 positions. Subsequent aerobic treatment with alkali results in the formation of a compound having a 10-formyl group which is susceptible to hydrolysis by dilute alkali (11, 12, 15, 16). In view of the instability of the intermediates involved in the degradation of CF to PGA, a recovery of even 25 per cent of the radioactivity of the enzymatically formed CF as formic acid indicates the probability that the C^{14} is located in the 5-formyl group of the isolated *citrovorum* factor.

DISCUSSION

The demonstration by Gordon *et al.* (17) that 10-formyl-PGA was approximately 30 times as effective as PGA in reversing the toxicity of a folic acid antagonist for *S. faecalis* directed attention to formylated derivatives of folic acid as a means for the metabolic transfer of formate. A great deal of evidence now indicates that derivatives of folic acid function in the transfer of single carbon units in several metabolic systems (18–22). The evidence that 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (CF, folinic acid-SF, leucovorin), in contrast to PGA, promoted the growth of *L. citrovorum* and counteracted the effect of the potent antagonists of PGA in various systems suggested that a reduced and formylated

derivative of PGA is closer to the functional derivative of this vitamin (1, 19).

Buchanan and Schulman (23) found that the exchange of carbon-2 of inosinic acid with labeled formate was increased by the addition of folic acid and, to a greater extent, by CF. However, it was not possible to conclude that, in the transfer of this 1-carbon unit, the formyl group of CF was itself transferred, although such a mechanism was postulated by the above authors as the rôle of CF in their system. In fact, studies by Elwyn *et al.* (24) indicated that in the synthesis of methyl groups from the β-carbon of serine the labilization of deuterium was less than that which would occur if the intermediate involved passed through the oxidation level of formate, and therefore a formyl-containing *citrovorum* factor could not be involved in the reaction. More recent studies by Elwyn and Sprinson (25) indicate that the pathway involved in the transfer of the β-carbon of serine into the 5-methyl of thymine differs from that involved in the transfer into the 2 and 8 positions of purines. Greenberg (26) has presented evidence that a formylated derivative of tetrahydro-PGA is involved in the incorporation of formate into inosinic acid, while the findings of Kisliuk and Sakami (27) suggest that a hydroxy methyl derivative of tetrahydro-PGA is involved in the biosynthesis of serine.

On the basis of recent evidence, it is unlikely that CF itself is an active cofactor (27–29). Metabolic alteration of CF to a compound capable of activating the transfer of formate for the formation of inosinic acid required the mediation of adenosinetriphosphate (29). The participation of activated formyl-labeled CF in such a system may permit a direct approach to the question of the actual transfer of this formyl group.

SUMMARY

Labeled *citrovorum* factor (CF) was formed by incubating cells of an A-Methopterin-resistant strain of *Streptococcus faecalis* with pteroylglutamic acid and formate-C^{14}. The isolated CF had twice the microbial potency of synthetic 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid, and appeared to be identical to this compound with respect to ultraviolet absorption spectrum and R_F on paper chromatography. Evidence is presented that the formyl carbon of CF can be derived from formate.

We wish to thank Dr. A. D. Welch for his interest and encouragement and Dr. C. E. Carter for assistance with the ion exchange chromatography. Pteroylglutamic acid and synthetic *citrovorum* factor (leucovorin) were generously supplied by the Calco Chemical Division of the American Cyanamid Company through the courtesy of Dr. J. M. Smith, Jr.

Addendum—More recently, it was found that CF derived from PGA by bacterial or liver preparations represents the stable product which is formed under *anaerobic* conditions by the non-enzymatic alteration of a labile precursor which has properties similar to those of 10-formyl-tetrahydropteroylglutamic acid (30).

BIBLIOGRAPHY

1. Jukes, T. H., *Federation Proc.*, **12,** 633 (1953).
2. Broquist, H. P., Kohler, A. R., Hutchison, D. J., and Burchenal, J. H., *J. Biol. Chem.*, **202,** 59 (1953).
3. Nichol, C. A., *J. Biol. Chem.*, **207,** 725 (1954).
4. Burchenal, J. H., Waring, G. B., and Hutchison, D. J., *Proc. Soc. Exp. Biol. and Med.*, **78,** 311 (1951).
5. Flynn, L. M., Williams, V. B., O'Dell, B. L., and Hogan, A. G., *Anal. Chem.*, **23,** 180 (1951).
6. Sauberlich, H. E., *J. Biol. Chem.*, **181,** 467 (1949).
7. Keresztesy, J. C., and Silverman, M., *J. Am. Chem. Soc.*, **73,** 5510 (1951).
8. Cosulich, D. B., Smith, J. M., Jr., and Broquist, H. P., *J. Am. Chem. Soc.*, **74,** 4215 (1952).
9. Nichol, C. A., Zakrzewski, S. F., and Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, **83,** 272 (1953).
10. Zakrzewski, S. F., and Nichol, C. A., *J. Biol. Chem.*, **205,** 361 (1953).
11. Pohland, A., Flynn, E. H., Jones, R. G., and Shive, W., *J. Am. Chem. Soc.*, **73,** 3247 (1951).
12. Cosulich, D. B., Roth, B., Smith, J. M., Jr., Hultquist, M. E., and Parker, R. P., *J. Am. Chem. Soc.*, **74,** 3252 (1952).
13. Keresztesy, J. C., and Silverman, M., *J. Biol. Chem.*, **183,** 473 (1950).
14. Bardos, T. J., Bond, T. J., Humphreys, J., and Shive, W., *J. Am. Chem. Soc.*, **71,** 3852 (1949).
15. May, M., Bardos, T. J., Barger, F. L., Lansford, M., Ravel, J. M., Sutherland, G. L., and Shive, W., *J. Am. Chem. Soc.*, **73,** 3067 (1951).
16. Cosulich, D. B., Roth, B., Smith, J. M., Jr., Hultquist, M. E., and Parker, R. P., *J. Am. Chem. Soc.*, **73,** 5006 (1951).
17. Gordon, M., Ravel, J. M., Eakin, R. E., and Shive, W., *J. Am. Chem. Soc.*, **70,** 878 (1948).
18. Shive, W., *Vitamins and Hormones*, **9,** 75 (1951).
19. Welch, A. D., and Nichol, C. A., *Ann. Rev. Biochem.*, **21,** 633 (1952).
20. Shive, W., *Federation Proc.*, **12,** 639 (1953).
21. Buchanan, J. M., and Wilson, D. W., *Federation Proc.*, **12,** 646 (1953).
22. Greenberg, G. R., *Federation Proc.*, **12,** 651 (1953).
23. Buchanan, J. M., and Schulman, M. P., *J. Biol. Chem.*, **202,** 241 (1953).
24. Elwyn, D., Weissbach, A., and Sprinson, D. B., *J. Am. Chem. Soc.*, **73,** 5509 (1951).
25. Elwyn, D., and Sprinson, D. B., *J. Biol. Chem.*, **207,** 467 (1954).
26. Greenberg, G. R., *J. Am. Chem. Soc.*, **76,** 1458 (1954).
27. Kisliuk, R. L., and Sakami, W., *J. Am. Chem. Soc.*, **76,** 1456 (1954).
28. Blakley, R. L., *Nature*, **173,** 729 (1954).
29. Greenberg, G. R., *Federation Proc.*, **13,** 221 (1954).
30. Nichol, C. A., Anton, A. H., and Zakrzewski, S. F., *Science*, **121,** 275 (1955).

REMOVAL OF THIOCTIC ACID FROM ENZYMES*

By GERALD R. SEAMAN and MARY DELL NASCHKE

(From the Carter Physiology Laboratory, University of Texas Medical Branch, Galveston, Texas)

(Received for publication, July 30, 1954)

The rôle of thioctic acid in the oxidative decarboxylation of α-keto acids is now well established (1, 2). This was accomplished largely by studies with microbial cells grown essentially free of the cofactor. In some instances (2) it has been possible to separate the coenzyme from microbial enzymes involved in thioctic acid metabolism by extensive purification procedures. Although the direct demonstration of the function of thioctic acid in vertebrate enzyme systems is lacking, the occurrence of large amounts of the cofactor in purified pyruvic (3) and α-ketoglutaric (4) oxidases from pigeon breast muscle and pig heart indicates (5) that the rôle of the cofactor in vertebrate systems parallels that in microbes. The factor is bound so tightly to the vertebrate enzymes that such procedures as dialysis, repeated washings, or ion exchange techniques do not dislodge it.

Experiments with the α-keto acid oxidases from the ciliated protozoan, *Tetrahymena pyriformis* S, indicate that thioctic acid can be readily removed from the enzyme by adsorption with alumina (6). It has since been recognized (7) that cofactor removal by this procedure requires the participation of an enzyme fraction which seems to catalyze the liberation of thioctic acid from protein-bound coenzyme in a reversible manner; the free thioctic acid which is liberated is then adsorbed on the alumina. Ammonium sulfate precipitation of pigeon liver acetone powder extracts separates a fraction which liberates the cofactor from enzymes. Since this fraction has not as yet been purified enough to ascertain the nature and specificity of the enzyme, the ammonium sulfate fraction has been termed (8) a "thioctic acid-splitting" fraction which contains "thioctic acid-splitting" activity.

Methods

The alumina treatments were carried out in conical centrifuge tubes. The enzyme solution was brought to room temperature and then rapidly stirred for 3 minutes with adsorption alumina. The adsorbent was used

* Aided by grants from the United States Public Health Service (No. G-3364), the National Vitamin Foundation, and by a contract between the Office of Naval Research and The University of Texas (NR160-160).

in a ratio of 1 gm. for each 100 mg. of protein. The tube was then cooled in an ice bath, and the alumina was allowed to settle. The supernatant fluid was pipetted and was then centrifuged at 2000 × *g* for 15 minutes at 4° to remove the remainder of the alumina. Treatment at room temperature effects more complete removal of thioctic acid than is accomplished at the lower temperatures used previously (6, 7).

Examination of different batches of alumina from various suppliers indicated that material with adsorptive capacities barely equivalent to a Brockmann characterization of II (9) is satisfactory; *i.e.*, from a mixture of Sudan yellow and methoxyazobenzene (2 mg. of each dye in 2 ml. of a mixture (1:4) of benzene and light mineral oil) at least half of the methoxyazobenzene is retained in a 10 × 1.5 cm. column which is packed to a height of 5 cm. Sudan yellow should not migrate more than 10 mm. from the top of the column. Development is accomplished with 20 ml. of a mixture (4:1) of benzene and light mineral oil.

Large mesh (80 to 200) adsorbent is routinely used directly from the manufacturer's container. Acid or alkali washings do not uniformly affect the ability to adsorb thioctic acid.

Following enzyme treatment, the adsorbed thioctic acid may be removed from the alumina with a mixture containing 89 per cent benzene, 5 per cent $CHCl_3$, 5 per cent ethanol, and 1 per cent butanol. Thioctic acid is extracted from the organic mixture by shaking with $\frac{1}{3}$ volume of 5 per cent aqueous $NaHCO_3$. Manometric assay (10) of the bicarbonate extracts indicates a recovery of 40 to 60 per cent of the thioctic acid removed from the enzyme. These assays are, however, only approximate, since the extracts contain oxidizable substrates. The enzymes were also assayed manometrically for thiotic acid before and after alumina treatment; the cofactor was liberated by hydrolysis in 6 N H_2SO_4 for an hour at 120°.

Analytical Methods—Pyruvate oxidation was measured by the rate of dismutation of pyruvate to acetyl phosphate, carbon dioxide, and lactate.[1]

(1) Pyruvate + DPN^+ + CoA $\rightleftarrows$ acetyl CoA + CO_2 + DPNH + H^+ (pyruvic oxidase)

(2) Acetyl CoA + phosphate $\rightleftarrows$ acetyl phosphate + CoA (phosphotransacetylase)

(3) Pyruvate + DPNH + H^+ $\rightleftarrows$ lactate + DPN^+ (lactic dehydrogenase)

(4) Sum, 2 pyruvate + phosphate $\rightleftarrows$ acetyl phosphate + CO_2 + lactate

Acetyl phosphate formation was determined by the hydroxamic acid method (11).

[1] The following abbreviations are used: oxidized and reduced diphosphopyridine nucleotide, DPN^+ and DPNH; coenzyme A, CoA; diphosphothiamine, DPT; glutathione, GSH.

The initial step in oxidation of α-ketoglutarate (Reaction 5), which is

(5) α-Ketoglutarate + DPN^+ + CoA ⇄ succinyl CoA + CO_2 + DPNH + H^+

analogous to Reaction 1 of pyruvate, was measured by following the reduction of DPN by the increase in optical density at 340 mμ in the Beckman DU spectrophotometer (12).

Preparation of Enzymes—T. pyriformis S was grown in mass culture in 21 liter Pyrex carboys containing 10 liters of 2 per cent "bacteriological peptone" (Nutritional Biochemicals Corporation), 0.5 per cent yeast extract (Nutritional Biochemicals), and approximately 5 parts per million of Antifoam A (Dow Corning Corporation). The carboys were well aerated on a vacuum line, and after 48 hours of growth at 25° ± 1.5° the cells were harvested by passage through a Sharples supercentrifuge. The collected cells were suspended in 0.01 M phosphate buffer, pH 7.4, and washed three times with the same buffer. The concentrate was ground with twice its weight of powdered quartz on a mechanical grinder for 30 minutes at 4° and extracted with 5 volumes of 0.01 M phosphate buffer, pH 7.4, for an additional 30 minutes. The suspension was clarified by centrifuging at 5000 × *g* for 15 minutes in the cold.

Pyruvic oxidase activity of *Streptococcus faecalis* was measured with extracts prepared according to Korkes *et al.* (13); the cells were grown in the complete AC.3 medium (10). Pyruvic oxidase from pigeon breast muscle was prepared as described by Jagannathan and Schweet (14) from muscle which had been stored at −20° for 8 to 12 months. The material obtained at Step 1 and at Step 6 of this procedure was examined. The dialyzed *S. faecalis* extracts added to incubation mixtures as a source of phosphotransacetylase and lactic dehydrogenase were prepared (13) from acetone powders of cells grown in synthetic, thioctic acid-deficient medium (10).

Purification of α-ketoglutaric oxidase followed the procedure described by Sanadi *et al.* (4). The specific activity of the ammonium acetate precipitate was 63, expressed as units per mg. of protein.

The thioctic acid-splitting fraction was prepared from extracts of pigeon liver acetone powder as described previously (7).

Results

Treatment of extracts of *Tetrahymena* with alumina removes large quantities of thioctic acid from the enzyme. This is accompanied by almost complete loss of activity. The activity of alumina-treated enzyme is almost entirely restored by the addition of 6,8-thioctic acid (Table I). Similar results are obtained with extracts of *S. faecalis* prepared from either lyophilized or acetone-dried cells (Table II).

The adsorption procedure, however, does not remove thioctic acid or

alter the pyruvic oxidase activity of phosphate extracts of washed pigeon breast muscle minces. However, extracts prepared from acetone powders of breast muscle, although containing only a fraction of the enzymatic

TABLE I

Effect of Alumina on Pyruvate Dismutation by Extracts of T. pyriformis

The incubation mixture contained, in 1.4 ml., 100 μmoles of Na pyruvate, 10 μmoles of $MgCl_2$, 0.22 μmole of DPT, 20 units of CoA, 10 μmoles of GSH, 0.1 μmole of DPN^+, dialyzed *S. faecalis* extract containing 0.28 mg. of protein as the source of phosphotransacetylase and lactic dehydrogenase, 100 μmoles of phosphate buffer, pH 7.4, and 10 mg. of *Tetrahymena* extract. Incubation was for 60 minutes at 32° in nitrogen.

Enzyme treatment and additions	Thioctic acid concentration of enzyme mixture	Acetyl phosphate formed
	γ	*μmoles*
None	0.370	1.1
" + 0.5 γ thioctic acid		1.0
Alumina-treated	0.021	0.1
" + 0.5 γ thioctic acid		1.3

TABLE II

Effect of Alumina Treatment on Pyruvate Dismutation by Extracts of S. faecalis

The incubation mixture contained, in 1.0 ml., 50 μmoles of phosphate buffer, pH 7.4, 2.4 μmoles of $MgCl_2$, 1.6 μmoles of $MnCl_2$, 11 μmoles of GSH, 0.15 μmole of DPN^+, 0.47 μmole of DPT, 28 units of CoA, 50 μmoles of Na pyruvate, and dialyzed *Streptococcus* extract containing 7 mg. of protein. Incubation was for 40 minutes at 38° under nitrogen.

Enzyme treatment and additions	Lyophilized cells		Acetone powder	
	Thioctic acid concentration of enzyme mixture	Acetyl phosphate formed	Thioctic acid concentration of enzyme mixture	Acetyl phosphate formed
	γ	*μmoles*	γ	*μmoles*
None	0.199	2.5	0.232	2.7
" + 0.5 γ thioctic acid		2.4		2.4
Alumina-treated	0.021	0.2	0.042	0.3
" + 0.5 γ thioctic acid		2.1		2.6

activity of the washed mince extracts, do respond to alumina treatment. The thioctic acid-splitting activity is thus separated from the minced preparation during the washing and extraction procedure. Addition of the thioctic acid-splitting fraction from pigeon liver to these phosphate ex-

TABLE III

Removal of Thioctic Acid from Pyruvic Oxidase of Pigeon Breast Muscle

The incubation mixture contained, in 1.0 ml., 50 μmoles of phosphate buffer, pH 7.4, 2.4 μmoles of $MgCl_2$, 1.6 μmoles of $MnCl_2$, 11 μmoles of GSH, 0.15 μmole of DPN^+, 0.47 μmole of DPT, 28 units of CoA, 50 μmoles of Na pyruvate, and dialyzed *S. faecalis* extract containing 0.24 mg. of protein as the source of phosphotransacetylase and lactic dehydrogenase. In Experiments 1 and 3, 7.8 mg. of protein of breast muscle extract of specific activity 0.3 were used. 30 mg. of enzyme protein were used in Experiment 2; the powder had a specific activity of <0.1. 10 mg. of 0 to 37 per cent liver fraction were used in Experiment 3. When indicated, the liver fraction was inactivated by heating at 70° for 6 minutes. Experiments 4 and 5 contained 0.316 mg. of protein of pyruvic oxidase, specific activity 42. In Experiment 5, 35 mg. of protein of the 0 to 37 per cent liver fraction were added. All incubations were carried out under nitrogen for 60 minutes at 38°.

Experiment No.	Enzyme fraction	Treatment	Thioctic acid content of added enzyme mixture	Acetyl phosphate formed
			γ	μmoles
1	Minced breast muscle extract	None	0.263	1.2
		" + 0.5 γ thioctic acid		1.1
		Alumina-treated	0.274	1.4
2	Extract of acetone powder of breast muscle	None	1.230	0.6
		" + 0.5 γ thioctic acid		0.6
		Alumina-treated	0.084	0.1
		" + 0.5 γ thioctic acid		0.5
3	Minced breast muscle extract + 0–37% liver fraction	None	0.330	1.3
		" + 0.5 γ thioctic acid		1.3
		Alumina-treated	0.024	0.2
		" + 0.5 γ thioctic acid		1.1
		Alumina-treated but liver fraction heat-inactivated	0.341	1.3
4	Pyruvic oxidase	None	0.178	1.7
		Alumina-treated	0.183	1.7
5	" " + 0–37% liver fraction	None	0.380	1.7
		" + 1 γ thioctic acid		1.6
		Alumina-treated	0.044	0.2
		" + 1 γ thioctic acid		1.8

tracts results in ready response to the alumina procedure for removal of the cofactor. Table III shows that the adsorption technique is also effective in removing thioctic acid from highly purified pyruvic oxidase from pigeon breast muscle (specific activity 42). Stimulation of dismutation activity is achieved by addition of thioctic acid to alumina-treated enzymes. The alumina procedure, in conjunction with the thioctic acid-splitting fraction, also removes the cofactor from purified α-ketoglutaric oxidase (Fig. 1).

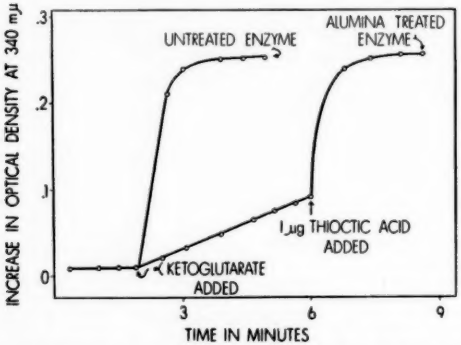


FIG. 1. Effect of alumina treatment of α-ketoglutaric oxidase on rate of reduction of DPN^+. The incubation mixture contained, in 3.0 ml., 75 units of CoA, 50 μmoles of GSH, 0.3 μmole of DPN, 100 μmoles of glycylglycine, pH 7.2, and enzyme mixture. The CoA and GSH were preincubated at room temperature for 15 minutes before the addition of the other components. After 2 minutes 5 μmoles of α-ketoglutarate were added to start the reaction. A cuvette containing all components except the substrate served as the blank. The enzyme mixture contained 0.33 mg. of protein of α-ketoglutaric oxidase, specific activity 66, and 10.9 mg. of 0 to 37 per cent liver fraction. The thioctic acid content of the untreated mixture was 0.263 γ of thioctic acid, and in the alumina-treated mixture the cofactor amounted to 0.016 γ.

The ready removal of thioctic acid from α-keto acid oxidase from *Tetrahymena*, *S. faecalis*, pigeon breast muscle, and pig heart by alumina adsorption attests to the general applicability of the method for removing the cofactor from enzymes with negligible inactivation of the enzyme protein. It may be anticipated that the procedure will allow the direct demonstration of thioctic acid participation in additional enzyme systems suspected of requiring the cofactor.

SUMMARY

Alumina treatment of extracts of *Tetrahymena pyriformis* and of *Streptococcus faecalis* removes thioctic acid from the enzyme proteins and is

accompanied by a decreased rate of pyruvate oxidation. Activity of the treated extracts is restored upon addition of thioctic acid.

The procedure, in the presence of an ammonium sulfate fraction from pigeon liver acetone powder extracts, also removes thioctic acid from purified pyruvic oxidase from pigeon breast muscle and from purified α-ketoglutaric oxidase from pig heart muscle.

The authors are indebted to Dr. I. C. Gunsalus for a culture of *S. faecalis* strain 10C1 and to Dr. E. L. R. Stokstad for generous supplies of thioctic acid.

BIBLIOGRAPHY

1. Reed, L. J., and DeBusk, B. G., *J. Am. Chem. Soc.*, **75**, 1261 (1953).
2. Gunsalus, I. C., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore, 545 (1954).
3. Schweet, R. S., and Cheslock, K., *J. Biol. Chem.*, **199**, 749 (1952).
4. Sanadi, D. R., Littlefield, J. W., and Bock, R. M., *J. Biol. Chem.*, **197**, 851 (1952).
5. Green, D. E., *Science*, **115**, 661 (1952).
6. Seaman, G. R., *Proc. Soc. Exp. Biol. and Med.*, **82**, 184 (1953).
7. Seaman, G. R., *J. Am. Chem. Soc.*, **76**, 1712 (1954).
8. Seaman, G. R., *Federation Proc.*, **13**, 731 (1954).
9. Brockmann, H., and Schodder, H., *Ber. chem. Ges.*, **74**, 73 (1941).
10. Gunsalus, I. C., Dolin, M. I., and Struglia, L., *J. Biol. Chem.*, **194**, 849 (1952).
11. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).
12. Sanadi, D. R., and Littlefield, J. W., *J. Biol. Chem.*, **201**, 103 (1953).
13. Korkes, S., Stern, J. R., Gunsalus, I. C., and Ochoa, S., *Nature*, **166**, 439 (1950).
14. Jagannathan, V., and Schweet, R. S., *J. Biol. Chem.*, **196**, 551 (1952).

METABOLISM OF RIBOSE-5-PHOSPHATE BY AZOTOBACTER VINELANDII*

By L. E. MORTENSON† and P. W. WILSON

(From the Department of Bacteriology, University of Wisconsin, Madison, Wisconsin)

(Received for publication, August 2, 1954)

Because ribose-5-phosphate (R-5-P) and sedoheptulose-7-phosphate (S-7-P) have been found free in cells of the azotobacter, the mechanism of synthesis of these compounds was sought. Previously, Horecker (1) and Wood and Schwerdt (2) demonstrated in yeast and *Pseudomonas fluorescens* that S-7-P was produced from R-5-P by an enzyme called transketolase. The R-5-P was first converted by an isomerase to ribulose-5-phosphate, which was then split into glyceraldehyde-3-phosphate (G-3-P) and a 2-carbon cocarboxylase complex of the oxidation level of glycolaldehyde. The 2-carbon fragment in turn condensed with R-5-P to produce S-7-P; the over-all reaction was the production of 1 molecule each of S-7-P and G-3-P from 2 molecules of R-5-P. Recently Horecker *et al.* (3) have shown that the S-7-P in turn is converted into tetrose phosphate and a dihydroxyacetone-enzyme complex that condensed with G-3-P to yield fructose-6-phosphate (F-6-P).

That enzyme preparations of the azotobacter also convert R-5-P into S-7-P and G-3-P will be shown in this paper. The finding of R-5-P and S-7-P in cells of the azotobacter indicates that this reaction is significant in its metabolism. Although the conversion of 6-phosphogluconate (6-PG) into ribulose-5-phosphate has been demonstrated in other organisms, it has not yet been established in this bacterium; in fact, all evidence suggests that the azotobacter splits almost quantitatively 6-PG into pyruvate and G-3-P (4). A possible source of R-5-P in this organism could be synthesis from G-3-P and acetate, as has been demonstrated in *Leuconostoc mesenteroides*.[1] This would explain the synthesis of some pentose by the azotobacter when incubated with 6-PG (5), since the products are G-3-P and pyruvate; pyruvate is readily converted to acetate by this organism.

* Supported in part by grants from the National Science Foundation and the National Institutes of Health.

† Predoctoral Fellow of the National Science Foundation. Present address, Chemical Department, Experimental Station, E. I. du Pont de Nemours and Company, Inc., Wilmington, Delaware.

[1] Communication by R. D. DeMoss at the meeting of the Society of American Bacteriologists, Pittsburgh (1954).

XUM

Methods

The methods for growing *Azotobacter vinelandii* O (*Azotobacter agile*, var. *vinelandii*) and preparing extracts are discussed elsewhere (6). Oxidation of R-5-P was carried out manometrically in a Warburg apparatus. All coenzymes used for these studies were obtained commercially; reduced diphosphopyridine nucleotide (DPNH) was produced by purified *Zwischenferment* from *A. vinelandii* when incubated with Mg^{++} and glucose-6-phosphate (G-6-P) (5). The barium salt of R-5-P (Nutritional Biochemicals Corporation) was converted into the potassium salt, and a solution containing 20 μmoles per ml. was prepared from the barium salt. W. A. Wood, University of Illinois, kindly supplied a sample of sedoheptulose.

R-5-P and S-7-P were assayed by reaction with orcinol; concentrations of the two when in mixtures were determined by simultaneous equations (7). G-3-P was determined by reaction with orcinol (8) and anthrone (4). Correction for S-7-P had to be made, since the latter compound reacts with anthrone.

The S-7-P and R-5-P were partially separated by elution from Dowex 1 resin (9). The production of S-7-P was confirmed by chromatographing the mixture before and after hydrolysis of the phosphate with two solvent systems: (*a*) ammonia-ethanol-water, (*b*) water-saturated phenol. The sedoheptulose was identified by spraying with the reagent of Bevenue and Williams (10), which is specific for heptuloses.

The quantities of free S-7-P and R-5-P in azotobacter cells were determined as follows: 30 gm. of cell paste (approximately 6 gm., dry weight) were broken at 0° in a Raytheon 10 kc. magnetostrictive oscillator, the protein removed by treatment with trichloroacetic acid, the débris and cations removed by Dowex 50 resin, and the whole made to pH 8.5 with NH_4OH. After this solution was introduced to a Dowex 1 column (H form), fractions were eluted according to directions of Khym and Cohn (9). Lowering the concentration of borate more gradually than specified by these authors produced a solution of S-7-P relatively free from R-5-P, but R-5-P never was obtained completely free from S-7-P. Further analysis of the S-7-P and R-5-P was made as outlined earlier in this section.

EXPERIMENTAL

Isolation of S-7-P and R-5-P from Azotobacter—Approximately 5 mg. of S-7-P and 1 mg. of R-5-P were obtained from 6 gm. of dry cells of *A. vinelandii*. The spectra of these compounds after reaction with orcinol are presented in Fig. 1. The low level of R-5-P compared to S-7-P suggested that R-5-P was converted into S-7-P as demonstrated in *P. fluorescens* (2). When R-5-P was incubated with an azotobacter preparation in glycylglycine buffer at pH 7.4, the disappearance of the peak at 680 mμ,

which results when R-5-P reacts with orcinol, together with the appearance of an increasing optical density from 520 to 600 mμ was observed (Fig. 2). Table I records the concentrations of S-7-P, R-5-P, and G-3-P at varying times during the incubation. Some hexose was also produced during the

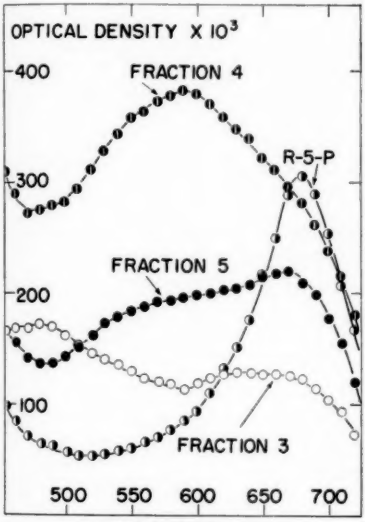


FIG. 1

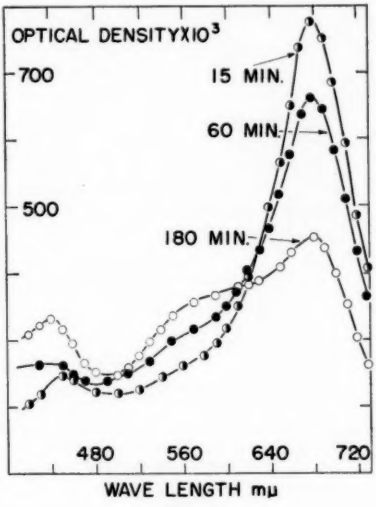


FIG. 2

FIG. 1. Orcinol spectra of the fractions of free phosphorylated compounds in *A. vinelandii* O after elution from a Dowex 1 column. 1.5 ml. of the respective fractions were mixed with 4.5 ml. of orcinol (12) and heated in a boiling water bath for 40 minutes. A spectroanalysis of the color formed was made in the Beckman spectrophotometer. All readings were made against a blank of the respective eluting solution. Fraction 3 is G-6-P, Fraction 4 is S-7-P and F-6-P, and Fraction 5 is S-7-P and R-5-P.

FIG. 2. Orcinol spectrum showing the loss of absorbancy at 680 mμ and a gain in absorbancy at 520 to 600 mμ when R-5-P was incubated with an azotobacter extract. The initial contents were 82 μmoles of R-5-P, 0.5 ml. of enzyme (23 mg. of protein), diluted to 5 ml. with 0.05 M glycylglycine buffer. Aliquots were taken periodically (0.025 ml. of 5 ml.), treated with orcinol, and the spectrum of the color produced recorded in a Beckman spectrophotometer against a blank without R-5-P.

reaction, but no careful analysis was made for it in this experiment. The incubation mixture was deproteinized, and paper chromatograms were prepared to confirm the presence of S-7-P. By the solvent system ammonia-ethanol-water (11), the following R_F values were recorded: unknown 0.64, sedoheptulose 0.74, and the mixture 0.64 and 0.74. The unknown

mixture was dephosphorylated by incubation with acid phosphatase, rechromatographed, and the R_F values now recorded were 0.75, 0.74, and 0.74, respectively. The R_F values of the dephosphorylated unknown, the known, and the mixture were 0.42 when water-saturated phenol was used as solvent. This agrees closely with the results of Wood and Schwerdt

TABLE I

*Stoichiometry of Conversion of R-5-P to S-7-P by A. vinelandii**

Time	R-5-P†	S-7-P	G-3-P
min.	*μmoles*	*μmoles*	*μmoles*
0	82.0	0.0	0.0
15	45.0	15.9	
60	34.0	23.8	17.6
180	20.3	31.2	16.0‡

* Incubation mixture as in Fig. 2.

† R-5-P and S-7-P were determined by reaction with orcinol and simultaneous equations at 680 and 570 mμ (9); G-3-P, by reaction with anthrone reagent (4).

‡ Contained some hexose.

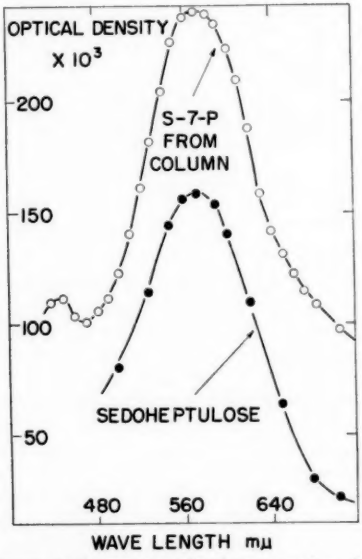


FIG. 3. Orcinol spectrum of the most purified preparation of S-7-P from a Dowex 1 column (9). 3 ml. of the fraction were concentrated to 1.5 ml. and 4.5 ml. of orcinol reagent added. The spectrum was recorded as in Fig. 6. A known sample of sedoheptulose was included for comparison.

(2). In addition a faint spot which moved rapidly and gave a blue color with the spray for heptulose was also noted; this might correspond to an epimer of sedoheptulose.

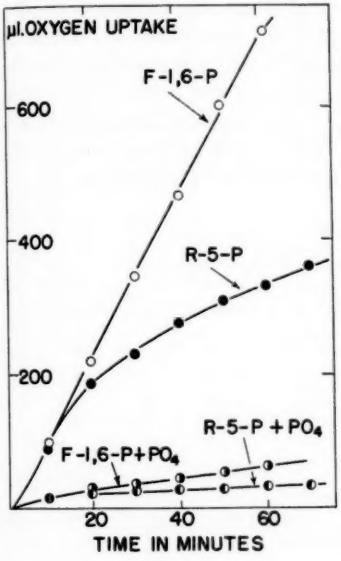


FIG. 4

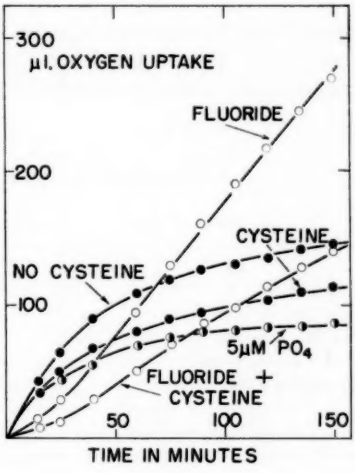


FIG. 5

FIG. 4. Effect of phosphate on R-5-P and fructose diphosphate oxidation. The reaction was measured by following oxygen uptake from air in a Warburg apparatus at 30°. Each vessel contained, in 3 ml. volume, 24 mg. of extract protein, 500 γ of DPN, 6 μmoles of Mg^{++}, 0.05 M glycylglycine, and 10 μmoles of R-5-P or 16 μmoles of F-1,6-P as designated. 100 μmoles of phosphate were added when testing for inhibition. The final pH of each vessel was adjusted to 7.4. 0.2 ml. of 20 per cent KOH was added to the center well to absorb the carbon dioxide evolved.

FIG. 5. Influence of fluoride and cysteine on the oxidation of R-5-P. All gas exchange was measured manometrically in a Warburg apparatus at 30° in air. All flasks contained 16.7 μmoles of R-5-P, 0.75 μmole of DPN, 6 μmoles of Mg^{++}, 24 mg. of extract protein, 0.05 M glycylglycine, and, when designated, 3 μmoles of cysteine, 3 μmoles of fluoride, or 5 μmoles of phosphate. The total volume and final pH were 3 ml. and 7.4, respectively. 0.2 ml. of 20 per cent KOH was added to the center well.

The remaining mixture was desalted and deproteinized, introduced to a Dowex 1 column, and the phosphorylated compounds were removed by elution with the specific eluents used by Khym and Cohn (9). The spectrum of the color of the most pure fraction of S-7-P after reaction with orcinol is given in Fig. 3.

Oxidation of R-5-P by Azotobacter—The oxidation of R-5-P by *A. vinelandii* O and the effect of M/30 and M/300 phosphate, fluoride, and cysteine on this oxidation are illustrated in Figs. 4 and 5. M/30 phosphate inhibited the oxidation 90 per cent; M/300 phosphate, 42 per cent. The addition of cysteine to the preparation (Fig. 5) greatly inhibited the oxidation of R-5-P, but the presence of fluoride stimulated it. Table II records the final oxygen consumed, the R-5-P which remained after oxidation, and the sedoheptulose which was produced and remained. In the presence of fluoride and absence of Mg^{++} and cysteine, little S-7-P was found, even though R-5-P was still available. The presence of cysteine inhibited the oxidation, but had no effect on S-7-P accumulation.

TABLE II

*Analytical Data on Oxidation of R-5-P by A. vinelandii**

Treatment	Oxygen uptake	Ribose left	Sedoheptulose
	μatom per μmole	*μmoles*	*μmoles*
Complete	0.75	0.30	3.1
+ cysteine	0.60	1.20	2.9
No Mg^{++} + F^-	1.40	1.50	0.1
+ F^- and cysteine	0.72	1.20	1.5
10 μmoles PO_4	0.44	5.20	1.9

* The reaction mixture of the flasks was as designated in Fig. 5. Ribose and sedoheptulose were measured by reaction with orcinol reagent. Final analyses were made after 150 minutes incubation.

That R-5-P or the products of its metabolism can enter into the tricarboxylic acid cycle is made probable by the results shown in Fig. 6. With succinate as a source of oxalacetate the oxidation of 5.0 μmoles of R-5-P was increased above the sum of the individual oxidations of succinate and R-5-P; this oxidation occurred only when cofactors were added: adenosinediphosphate, adenosinetriphosphate, coenzyme A, diphosphopyridine nucleotide, triphosphopyridine nucleotide, and Mg^{++}. Succinate as the sole substrate was oxidized essentially to completion. Malonate greatly inhibited the oxidation, the total oxygen consumption being lower than that of either R-5-P or succinate alone.

If R-5-P is converted into ribulose-5-phosphate and the latter in turn split into G-3-P and a 2-carbon fragment as in other organisms, then the reduction of DPN by triosephosphate dehydrogenase would be ideal for demonstrating this system. Although no reduction occurred when DPN was added to the enzyme and R-5-P in the presence of adenosinediphosphate, rapid oxidation of reduced DPN occurred when this compound was

supplied (Fig. 7). This oxidation of DPNH did not occur in the absence of R-5-P, but did occur when F-1,6-P was added (5).

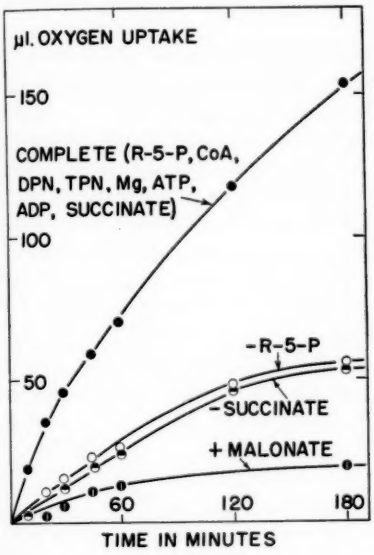


FIG. 6

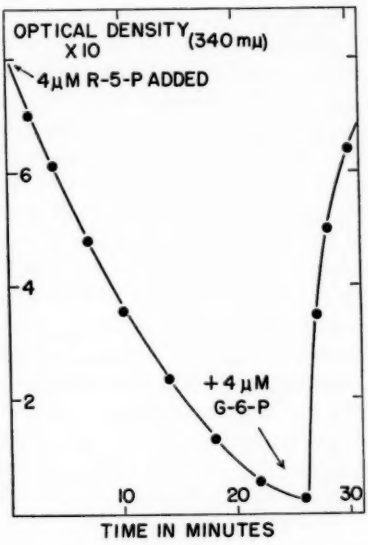


FIG. 7

FIG. 6. Oxidation of R-5-P and the influence of cofactors and succinate on the oxidation. Components of the Warburg flasks, 16 mg. of enzyme protein, 1 unit of coenzyme A, 500 γ of DPN, 100 γ of triphosphopyridine nucleotide, 6 μmoles of Mg^{++}, 0.5 μmole of adenosinetriphosphate, 0.5 μmole of adenosinediphosphate, 1 μmole of succinate, and 4.6 μmoles of R-5-P. The final contents were made to 3 ml. with 0.05 M glycylglycine buffer. Components of the complete system were eliminated as designated. Malonate (3 μmoles) was added to one flask containing the complete system. Oxygen consumption was followed in a Warburg apparatus at 30°. The center well contained 0.2 ml. of 20 per cent KOH.

FIG. 7. Oxidation of DPNH by the enzyme plus added R-5-P. Beckman cuvettes contained in 3.0 ml. 0.38 μmole of DPNH, 3 mg. of enzyme protein, 6 μmoles of Mg^{++}, 0.05 M glycylglycine buffer. The final pH was 7.6. At 0 minute 4 μmoles (0.1 ml.) of R-5-P were added, and the oxidation of DPNH observed. At 27 minutes 4 μmoles (0.1 ml.) of G-6-P were added to demonstrate the presence of *Zwischenferment* in the preparation and that the DPNH had been oxidized, not destroyed. Without R-5-P no oxidation occurred.

DISCUSSION

The evidence presented is consistent with the view that R-5-P is converted into S-7-P and G-3-P by *A. vinelandii* O. This is substantiated by the stoichiometry of the reaction: 82 μmoles of R-5-P were converted into

31.3 μmoles of S-7-P, 20.3 μmoles of R-5-P remaining. Since 2 molecules of R-5-P are required to yield 1 molecule of S-7-P and 1 molecule of G-3-P, the S-7-P formed from the R-5-P that disappeared is in excellent agreement with the system suggested.

The G-3-P found at 60 minutes (17.6 μmoles) agreed fairly well with the stoichiometry of the reaction, as 23.8 μmoles of S-7-P were formed. However, at 180 minutes only 16 μmole equivalents of G-3-P were found. Since the preparation contained minute amounts of DPN, it was suspected that either (*a*) the G-3-P underwent a dismutation, 1 molecule being oxidized to 3-phosphoglyceric acid, while another after isomerization to dihydroxyacetone phosphate was reduced to α-glycerophosphate, or (*b*) some G-3-P was converted to dihydroxyacetone phosphate which in turn condensed with G-3-P to yield F-1,6-P. The former view was substantiated by the rapid oxidation of DPNH in the presence of R-5-P and F-1,6-P (source of dihydroxyacetone phosphate). α-Glycerophosphate yields a spectrum with anthrone and orcinol different from that of G-3-P, and its optical density, after reaction with these reagents at the same concentration and at the wave-length used for determination of G-3-P, is less. This would account for the low recovery of G-3-P; hexose production would also decrease the expected yield of G-3-P as assayed by these methods. Low yields of G-3-P also have been found with animal extracts (7).

The further metabolism of sedoheptulose has not been studied directly, but the results observed during R-5-P oxidation in the presence of various inhibitors indicated that it was converted partly into F-6-P and tetrose phosphate, as has been suggested by Horecker *et al.* (3). F-6-P has been shown to be easily converted into G-6-P by *A. vinelandii*, the G-6-P in turn oxidized to 6-PG, and the 6-PG split into pyruvate and G-3-P (5). Several points of evidence suggest this as the most likely possibility. First, phosphate strongly inhibits R-5-P oxidation, indicating that the oxidation proceeds through *Zwischenferment.* This evidence also strongly suggests that the oxidation does not proceed through G-3-P dehydrogenase, since the latter reaction requires phosphate. Second, fluoride increases the oxidation of R-5-P, and, since fluoride inhibits enolase, it would prevent oxidation of G-3-P after its initial oxidation to phosphoglyceric acid. The fact that more than a one-step oxidation of R-5-P occurred in the presence of fluoride and that the rate of oxidation had not fallen when the readings were stopped indicates that the oxidation does not go through this system. This view is further supported, since phosphoglyceric acid was not oxidized by these preparations in the presence of fluoride and only slightly in its absence. Finally, some hexose appeared to be formed by further incubation of the mixture; the definite identification of the hexose has not been made.

SUMMARY

Cells of *Azotobacter vinelandii* O contain free ribose-5-phosphate and sedoheptulose-7-phosphate in the ratio of approximately 1:3. Cell-free extracts of this organism convert ribose-5-phosphate to sedoheptulose-7-phosphate and triose phosphate. Further metabolism of the triose phosphate and sedoheptulose-7-phosphate results in the formation of a hexose phosphate, the hexose of which has not been characterized. In experiments in which sedoheptulose-7-phosphate is not further metabolized, the triose phosphate disappears, and some hexose is produced.

BIBLIOGRAPHY

1. Horecker, B. L., and Smyrniotis, P. Z., *J. Am. Chem. Soc.*, **74**, 2123 (1952).
2. Wood, W. A., and Schwerdt, R. F., *J. Biol. Chem.*, **206**, 625 (1954).
3. Horecker, B. L., Gibbs, M., Klenow, H., and Smyrniotis, P. Z., *J. Biol. Chem.*, **207**, 393 (1954).
4. Mortenson, L. E., Thesis, University of Wisconsin (1954).
5. Mortenson, L. E., and Wilson, P. W., *Arch. Biochem. and Biophys.*, in press.
6. Wilson, P. W., and Knight, S. G., Experiments in bacterial physiology, Minneapolis (1952).
7. Horecker, B. L., *J. Cell. and Comp. Physiol.*, **41**, 137 (1953).
8. Scott, D. B. M., and Cohen, S. S., *J. Cell. and Comp. Physiol.*, **38**, 173 (1951).
9. Khym, J. X., and Cohn, W. E., *J. Am. Chem. Soc.*, **75**, 1153 (1953).
10. Bevenue, A., and Williams, K. T., *Arch. Biochem. and Biophys.*, **34**, 225 (1951).
11. Bandurski, R. S., and Axelrod, B., *J. Biol. Chem.*, **193**, 405 (1951).
12. Brown, A. H., *Arch. Biochem.*, **11**, 269 (1946).

GLYCOGEN REGENERATION IN VIVO

By MARJORIE R. STETTEN and DeWITT STETTEN, Jr.

(From the Division of Nutrition and Physiology, The Public Health Research Institute of the City of New York, Inc., New York, New York, and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland)

(Received for publication, September 23, 1954)

A study of the mechanism of glycogen "turnover" in the tissues of the intact rat has been the subject of an earlier communication (1). Experiments were reported in which glycogen samples were procured from carcasses and livers of rats 3 to 48 hours after intraperitoneal injection of glucose-C^{14}. Each glycogen sample, after assay for C^{14}, was digested with malt β-amylase, and the distribution of isotope between the maltose and limit dextrin, LD (glycogen, β-amylase), was determined. It was found that, in general, the isotope was not uniformly distributed throughout the glycogen molecule, and that, at early time intervals, C^{14} was present in higher concentration in the peripherally situated (maltose) glucosidic residues. After about 12 hours, in liver glycogen, the specific activity of the centrally situated (LD) glucosidic residues exceeded that of the periphery, whereas in carcass glycogen, even after 48 hours, the maltose liberated by β-amylase was more radioactive than the limit dextrin.

The further exploration of this biological inhomogeneity of glycogen has required that the glycogen molecule be subjected not merely to a bisection, but also to a more detailed dissection. Such a dissection is rendered possible by the alternate application of phosphorylase and amylo-1,6-glucosidase, as described by Cori and Larner (2). The initial treatment of glycogen with crystalline rabbit muscle phosphorylase yields a limit dextrin, LD (glycogen, phosphorylase), termed LD-1. This product, after purification, is treated with crude rabbit muscle amylo-1,6-glucosidase in the absence of phosphate. The enzyme is then destroyed, and phosphate and phosphorylase are added, yielding a second limit dextrin, LD-2. Repetition of these treatments then yields a third product, LD-3. The extent of digestion accomplished at each step is measured by the sum of glucose + glucose-1-phosphate produced.

An initial series of degradations was carried out on a sample of rabbit muscle glycogen procured 6 hours after intravenous injection of glucose-C^{14}. Samples of glycogen from rat liver and rat carcass, selected from those used in the previous study (1), were then subjected to similar stepwise degradations, and successive limit dextrins were isolated and analyzed.

XUM

EXPERIMENTAL

Glycogen Samples—A well fed adult male rabbit weighing 3.68 kilos was injected intravenously with 100 μc. of uniformly labeled glucose-C^{14} with a specific activity of 0.476 μc. per mg. (Nuclear Instrument and Chemical Corporation, Chicago, Illinois.) Pellets and water were given *ad libitum*. 6 hours after the injection, the rabbit was killed by a blow on the head and liver and muscle glycogen isolated, purified, and analyzed as previously described (3, 4). The muscle glycogen, which contained 950 c.p.m. per milliatom of C, was used for enzymatic degradation studies.

The rat glycogen samples were isolated from animals killed 6, 12, 24, and 48 hours after receiving an intraperitoneal injection of a solution of 1 mmole of glucose-C^{14} per 100 gm. of body weight. The liver glycogens were those designated as C, H, I, and K and the carcass glycogens as G, H, J, and K in the preceding paper (1).

Enzymatic Degradations—The phosphorylase used was prepared by the method of Green and Cori (5).

180 mg. of anhydrous muscle glycogen of rabbit were incubated for 18 hours at 32° with phosphorylase in a total volume of 100 cc. of phosphate buffer of pH 6.8, containing a few mg. of adenylic acid. Glucose was determined by the method of Schales and Schales (6) on an aliquot of the solution after removal of protein and polysaccharide by the $HgCl_2$-HCl procedure (2) and acid hydrolysis of glucose-1-phosphate. Limit dextrin 1 was precipitated from the remainder of the solution by the addition of 2 volumes of ethanol. After removal of anions by means of Amberlite IRA-400 resin in aqueous solution, the limit dextrin was purified by successive alcohol precipitations from dilute alkali, cold 5 per cent trichloroacetic acid, and at least twice from water. The resulting LD-1 was found to be free of inorganic phosphate. Retreatment of LD-1 with phosphorylase in phosphate buffer resulted in the liberation of only a small amount of additional glucose-1-phosphate.

The phosphate-free LD-1 was incubated for 4 hours in citrate buffer of pH 7 with a crude solution of amylo-1,6-glucosidase which had been prepared and freed of α-amylase by the method described by Cori and Larner (2). After removal of the glucosidase, the polysaccharide solution was again treated with phosphorylase in phosphate buffer and LD-2 isolated and purified. In the same way, LD-3 was prepared and the amount of glucose + glucose-1-phosphate split off at each step was determined.

Starting with the four rat liver glycogens and four rat carcass glycogens, similar alternating digestions with phosphorylase and amylo-1,6-glucosidase, isolations of LD-1, LD-2, and LD-3, and analyses were carried out.

β-Amylase degradations were done as already described and β-amylase limit dextrins isolated (1).

Isolation of Glucose—Supernatant solutions from the first alcoholic precipitations of limit dextrins were evaporated to small volumes, acidified, and heated to hydrolyze glucose-1-phosphate, and from these solutions glucose was isolated as glucosazone. Glucosazone samples were recrystallized from aqueous alcohol and analyzed for C^{14} content.

RESULTS AND DISCUSSION

A stepwise degradation was conducted upon a sample of muscle glycogen obtained from a rabbit 6 hours after intravenous injection of uniformly labeled glucose-C^{14}. Three limit dextrins were procured by repetitive alternating treatment with phosphorylase and amylo-1,6-glucosidase, and, for comparison, another limit dextrin was made by exhaustive treatment of the original glycogen sample with β-amylase. In Table I are recorded the specific activities of the several polysaccharide samples, together with the values of the per cent of the initial glycogen which had been eliminated by enzymatic digestion in the course of preparation of each limit dextrin. These latter values have been computed from determinations of sugar liberated in the preparation of each limit dextrin. LD (β-amylase) is considered apart from the phosphorylase limit dextrins and is calculated with reference to the original glycogen from which it was directly obtained. The findings indicate that, as the glycogen molecules in this sample are progressively attacked from the non-reducing ends of the arboreal structure, limit dextrins of successively lower specific activities are obtained. Of the glucosidic residues in the periphery of the glycogen molecule, a large fraction is derived from the injected labeled glucose. In the center of the molecule, exemplified by LD-3, the corresponding fraction is far smaller.

From the data in Table I, the specific activity of each successive tier of glucosidic residues in the glycogen molecule may be calculated. Thus the average relative specific activity of glucose residues eliminated by the first treatment with phosphorylase (P-1) is equal to

$$\frac{10{,}000 - 4300}{37.9} = 150 \text{ c.p.m. per milliatom C (relative to 100 c.p.m. per milliatom C in glycogen)}$$

while that of the glucose in the second tier (P-2) eliminated from LD-1 by treatment with amyloglucosidase followed by phosphorylase becomes

$$\frac{4300 - 1740}{63.3 - 37.9} = 101 \text{ c.p.m. per milliatom C}$$

The corresponding quantities, calculated from the specific activities of glucosazone samples isolated after digestion of glycogen and of LD-1 with phosphorylase, were 158 and 105 c.p.m. per milliatom of C (relative to 100 c.p.m. per milliatom of C in glycogen).

From the results of such computations, Fig. 1 has been constructed wherein the height of each block represents relative specific activity, the width the relative amount of glucose contained, and the area equals the total counts per minute in that tier of the glycogen molecule. From Fig. 1, it will clearly be seen that the specific activities of glucose residues in this sample of glycogen decrease systematically and apparently fairly

TABLE I

Distribution of Radioactivity in Rabbit Muscle Glycogen 6 Hours after Intravenous Injection of Glucose-C^{14}

Sample	Specific activity	Relative specific activity*	Glycogen digested, cumulative	Relative total activity in polysaccharide†
	c.p.m. per milliatom C		*per cent*	*c.p.m.*
Glycogen	950	100	0	10,000
LD-1	657	69.2	37.9	4,300
LD-2	449	47.3	63.3	1,740
LD-3	284	29.9	77.7	667
LD (β-amylase)	582	61.3	44.0	3,430

* Expressed as per cent of specific activity of glycogen.

† The values in this column are obtained from the following expression: relative specific activity × (100 minus per cent of glycogen digested).

smoothly as one approaches the reducing end of the molecule from the peripheral non-reducing termini.

Four samples of rat carcass glycogen which had previously been studied by the β-amylase method (1) have now been degraded by the phosphorylase-amyloglucosidase technique. Three limit dextrins were serially prepared from each glycogen sample and the analytical findings are presented in Table II. At each time interval studied, in the case of carcass glycogen, a decreasing specific activity of successive limit dextrins was encountered. This decrease was more abrupt at 6 hours, when LD-3 exhibited 45 per cent of the specific activity of the glycogen, and was more gradual at 24 and 48 hours, when LD-3 was about 70 per cent as radioactive as the original glycogen. No limit dextrin was encountered which was more radioactive than the polysaccharide from which it was prepared. The yield of monosaccharide at each step, in this series of degradations, was lower than had been anticipated on the basis of our own

experience with glycogen from other sources and the reports of others (7). This may have resulted from a partial loss of activity of the phosphorylase preparation employed.

The glycogen samples obtained from the livers of four rats have likewise been studied by the present technique. These samples again are materials previously studied by the β-amylase method (1). The analytical data relating to the limit dextrins procured from each of these glycogen samples are presented in Table III.

The distribution of isotope in glycogen of liver 6 hours after injection of glucose-C^{14} resembles qualitatively that observed in the samples of muscle glycogen previously described. The specific activity of each successive

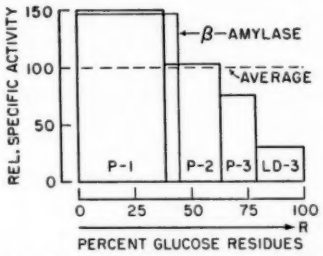


FIG. 1. Distribution of radioactivity in rabbit muscle glycogen 6 hours after injection of glucose-C^{14}. The ordinates represent the specific activities (counts per minute per milliatom of C) of the tiers of glucosidic residues relative to 100 c.p.m. per milliatom of glycogen carbon. The abscissas represent the percentages of glucose residues digested by the successive treatments of glycogen with enzymes. *R* represents the reducing end of the glycogen molecule. For calculations and significance of P-1, P-2, etc., see the text.

limit dextrin obtained from this sample is lower than that of the polysaccharide from which it was prepared. The series of limit dextrins prepared from the 12 hour rat liver glycogen sample shows about the same specific activity. By the 24th, and more strikingly, by the 48th hour, the sequence of specific activities in limit dextrins has become inverted. Thus in the products derived from rat liver glycogen 48 hours after injection of glucose-C^{14}, the specific activities are LD-3 > LD-2 > LD-1 > glycogen.

From the data in Tables I, II, and III, the relative specific activities in the successive tiers of glucosidic residues of each glycogen sample have been calculated and are presented in Table IV.

In order to describe more precisely the meaning of these data, Figs. 2 and 3 have been constructed with the same coordinates described in relation to Fig. 1. The dissection of rat carcass glycogen (Fig. 2) obtained 6 hours after intraperitoneal injection of glucose reveals that the highest

specific activity occurs in the peripheral (P-1) tier, and the lowest specific activity is found in the center (LD-3). With the passage of time, a slight decline in the specific activity of P-1 and a slight rise in that of LD-3

TABLE II

Distribution of Radioactivity in Rat Carcass Glycogen after Intraperitoneal Injection of Glucose-C^{14}

Sample	Specific activity	Relative specific activity*	Glycogen digested, cumulative	Relative total activity in polysaccharide*
	6 hrs.			
	c.p.m. per milliatom C		*per cent*	*c.p.m.*
Glycogen	1115	100	0	10,000
LD-1	828	74.2	27.9	5,350
LD-2	626	56.1	44.2	3,130
LD-3	504	45.2	50.3	2,250
	12 hrs.			
Glycogen	282	100	0	10,000
LD-1	210	74.5	27.2	5,420
LD-2	151	53.5	43.9	3,000
LD-3	147	52.1	50.3	2,590
	24 hrs.			
Glycogen	259	100	0	10,000
LD-1	238	91.9	27.5	6,660
LD-2	207	79.9	45.2	4,380
LD-3	188	72.6	52.9	3,420
	48 hrs.			
Glycogen	647	100	0	10,000
LD-1	590	91.2	26.8	6,670
LD-2	561	86.7	44.7	4,790
LD-3	453	70.0	56.5	3,050

* See foot-notes to Table I.

occur, but, at all times studied, the center of the molecule is the least radioactive portion.

From the studies of rat liver glycogen (Fig. 3), it may be seen that, 6 hours after injection of glucose, the peripheral tier (P-1) of the polysaccharide is again richest in isotope, and qualitatively this sample of glycogen

resembles that secured from rabbit muscle (see Fig. 1). After the passage of 12 to 24 hours, the several tiers of the liver glycogen samples studied all exhibit about the same radioactivity. By the 48th hour, a reversal

TABLE III

Distribution of Radioactivity in Rat Liver Glycogen after Intraperitoneal Injection of Glucose-C^{14}

Sample	Specific activity	Relative specific activity*	Glycogen digested, cumulative	Relative total activity in polysaccharide*
	6 hrs.			
	c.p.m. per milliatom C		*per cent*	*c.p.m.*
Glycogen	442	100	0	10,000
LD-1	328	74.4	34.0	4,910
LD-2	194	43.5	58.1	1,820
LD-3	103	23.4	73.9	610
	12 hrs.			
Glycogen	785	100	0	10,000
LD-1	814	104	35.0	6,760
LD-2	845	108	58.7	4,460
LD-3	768	97.8	72.6	2,680
	24 hrs.			
Glycogen	173	100	0	10,000
LD-1	174	101	37.5	6,300
LD-2	178	103	61.0	4,020
LD-3	202	117	75.8	2,830
	48 hrs.			
Glycogen	65	100	0	10,000
LD-1	75	115	34.8	7,500
LD-2	90	139	59.9	5,570
LD-3	97	149	75.1	3,710

* See foot-notes to Table I.

has occurred and now the periphery of the molecule (P-1) is the least radioactive portion, and specific activity is seen to increase as the reducing end of the molecule is approached.

From the present results, a picture of the mechanism of glycogen turnover *in vivo* emerges. The regeneration of glycogen proceeds by a rapid

TABLE IV

Relative Specific Activity in Successive Tiers Split from Glycogen Samples

From the specific activities found in each limit dextrin, the specific activity relative to 100 c.p.m. per milliatom of C in glycogen has been calculated (see the text). P-1, P-2, and P-3 represent the glucose eliminated upon successive treatments with phosphorylase and amylo-1,6-glucosidase. LD-3 is the limit dextrin remaining after the third treatment.

Tier	Rabbit	Rat							
	Muscle glycogen	Liver glycogen				Carcass glycogen			
	6 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.
P-1	150	150	93	99	72	167	168	121	124
P-2	101	128	97	97	78	136	145	129	105
P-3	75	77	128	80	122	144	64	125	147
LD-3	30	23	98	117	149	45	52	73	70

process wherein new glucosidic residues are added to the peripheral, nonreducing termini of preexisting glycogen molecules. The presumed source of these glucosidic residues is glucose-1-phosphate; the presumed catalyst is phosphorylase (8). By a second and slower process, glucose residues

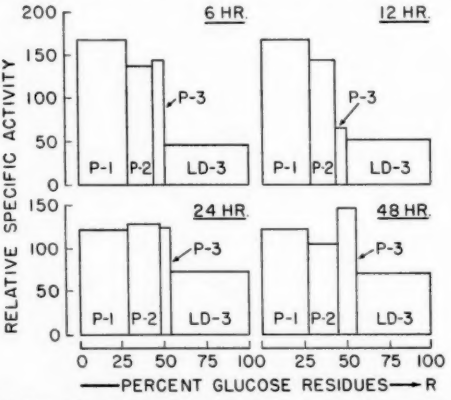


FIG. 2. Distribution of radioactivity in rat carcass glycogen samples at various times after injection of glucose-C^{14}. The ordinates represent the specific activities (counts per minute per milliatom of C) of the tiers of glucosidic residues relative to 100 c.p.m. per milliatom of glycogen carbon. The abscissas represent the percentages of glucose residues digested by the successive treatments of glycogen with enzymes. *R* represents the reducing end of the glycogen molecule. For calculations and significance of P-1, P-2, etc., see the text.

initially situated in peripheral tiers of the molecule of glycogen find their way into more centrally situated layers, presumably by the action of "branching enzyme." This has been termed amylo-(1,4 → 1,6)-transglucosidase by Larner who, using partially purified enzymes, has demonstrated the entry of glucosidic residues, initially in the outer tier, into an inner tier of glycogen (9). The present study suggests that the branching process is less active in muscle than in liver.

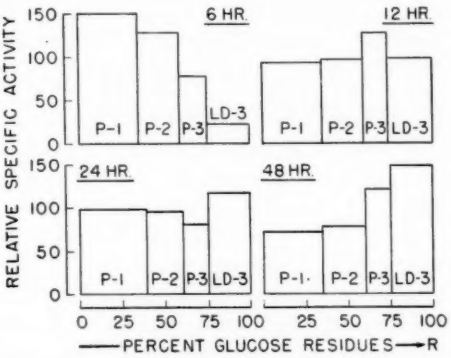


FIG. 3. Distribution of radioactivity in rat liver glycogen samples at various times after injection of glucose-C^{14}. The ordinates represent the specific activities (counts per minute per milliatom of C) of the tiers of glucosidic residues relative to 100 c.p.m. per milliatom of glycogen carbon. The abscissas represent the percentages of glucose residues digested by the successive treatments of glycogen with enzymes. *R* represents the reducing end of the glycogen molecule. For calculations and significance of P-1, P-2, etc., see the text.

SUMMARY

Glycogen samples have been secured from rabbit muscle, rat liver, and rat carcass at various times after injection of glucose-C^{14}. Each sample was degraded by alternate treatment with phosphorylase and amylo-1,6-glucosidase, yielding a series of limit dextrins. The radioactivity of these dextrins has been measured and compared with that in the original glycogen.

In samples collected 6 hours after injection, the peripheral tier of glucose residues of glycogen was the most radioactive. With the passage of time, the relative specific activity tended to decline in the periphery, but to rise in the center of the glycogen molecule. In rat liver glycogen, this process led, in 48 hours, to a reversed situation wherein the periphery of the molecule was the least and the center the most radioactive.

The relation of these findings to the mechanism of glycogen regeneration *in vivo* is discussed.

BIBLIOGRAPHY

1. Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.*, **207,** 331 (1954).
2. Cori, G. T., and Larner, J., *J. Biol. Chem.*, **188,** 17 (1951).
3. Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.*, **187,** 241 (1950).
4. Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.*, **193,** 157 (1951).
5. Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **151,** 21 (1943).
6. Schales, O., and Schales, S. S., *Arch. Biochem.*, **8,** 285 (1945).
7. Larner, J., Illingworth, B., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **199,** 641 (1952).
8. Cori, C. F., Cori, G. T., and Green, A. A., *J. Biol. Chem.*, **151,** 39 (1943).
9. Larner, J., *J. Biol. Chem.*, **202,** 491 (1953).

THE DISPOSITION OF TAURINE-S^{35} AND TAUROCHOLATE-S^{35} IN THE RAT: DIETARY INFLUENCES*

By OSCAR W. PORTMAN† and GEORGE V. MANN‡

(From the Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts)

(Received for publication, September 28, 1954)

A series of observations made in this laboratory indicated that the sulfur amino acids cystine and methionine are involved in cholesterol metabolism in a manner other than by lipotropic mechanisms. The *Cebus* monkey, the rat, and the mouse fed diets deficient in sulfur amino acids and high in cholesterol, but with adequate quantities of choline, developed a marked hypercholesterolemia (1, 2). The feeding of cystine or methionine prevented or abolished this hypercholesterolemia in monkeys; cystine and methionine were partially effective in preventing the hypercholesterolemia of rats produced by similar dietary means (2). Furthermore, taurine has consistently produced a significant lowering of serum cholesterol levels in hypercholesterolemic *Cebus* monkeys.[1] In elaborating the mechanisms of these observations, we have investigated the effect of sulfur amino acid deficiency and cholesterol feeding on taurine and bile acid metabolism. This approach was of particular interest since methionine, cysteine, sulfate, and glutathione (3–6) have each been demonstrated to be taurine precursors, and taurine has been shown to be conjugated and excreted with bile acids in many mammalian species (7).

The conversion of fed cholesterol to cholate has been demonstrated in several species (8, 9). Byers *et al.* (10) reported that bile duct ligation or cholate feeding delayed the elimination of cholesterol supplied to the rat and resulted in increased plasma cholesterol levels. Bergström and Norman (11) found taurocholic acid to be the principal steroid conjugate in rat bile, and Bergström *et al.* (12) have demonstrated the conjugation of taurine and cholate by rat liver slices *in vitro*.

Foster, Hooper, and Whipple (13) had previously shown that taurine

* This work was supported in part by grant No. H136C3 of the National Heart Institute, National Institutes of Health, Bethesda, Maryland, the Life Insurance Medical Research Fund, New York, and the Albert and Mary Lasker Foundation, New York.

† Postdoctoral Fellow of the Life Insurance Medical Research Fund during part of this work.

‡ A part of this work was done during tenure of an Established Investigatorship of the American Heart Association.

[1] Mann, G. V., unpublished data.

feeding did not significantly increase bile acid excretion in the dog. Hawkins *et al.* (14) reported that the ratio of conjugated to unconjugated bile acids excreted into the bile by dogs could shift markedly in the absence of taurine precursors. These data suggested that the conversion of taurine precursors to taurine and its conjugation with bile acids is not an obligatory reaction in the dog. On the other hand, it has been demonstrated that when cholic acid was fed to the growing rat the animal's requirement for organic sulfur was increased (15).

We first studied the routes and extent of excretion and tissue fixation of administered taurine-S^{35} and the biliary excretion of bile acids in normal rats. Similar processes were then studied in rats prepared with diets low in sulfur amino acids and with different dietary levels of fat and cholesterol. The metabolism of biosynthetic taurocholate-S^{35} was studied in normal rats.

Methods

Preparation of Animals—Male albino rats weighing about 350 gm. were used. During the period prior to experimental procedures rats were caged in groups and supplied with food and water *ad libitum*. Animals were prepared with the experimental diets (see Table I) for periods of 45 to 60 days before bile duct cannulation was undertaken. The sulfur-deficient animals then showed definite evidence of deprivation in loss of hair and in weight stabilization or loss. Bile duct cannulation was accomplished under ether anesthesia. An 8 cm. length of 0.61 mm. (outer diameter) polyethylene tubing was inserted proximally into the bile duct. A similar cannula was inserted into the bile duct distally and threaded posteriorly until 5 mm. extended into the duodenum. The cannulas were tied in place and the free ends were brought outside and connected by a snugly fitted polyethylene sleeve. This arrangement furnished a means for either collection of bile or resupply to the duodenum. Thoracic duct cannulation was carried out by the method of Bollman *et al.* (16). The animals were housed in wire tube restraining cages during the collection periods, and provision was made for a supply of diet and water, as well as for the collection of urine and feces. Collections of body excretions were begun 2 hours after completion of the cannulation and continued for 24 hours in 6 hour fractions. A criterion of acceptability of data was that of animal survival for 24 hours or longer after the completion of the collection.

Synthesis of Radio Compounds—Taurine-S^{35} was synthesized by a modification of the method of Cortese (17, 18); α-bromoethylamine hydrobromide was allowed to react with sodium sulfite-S^{35} in a molar ratio of 1.5:1.[2]

[2] Sulfurous acid-S^{35} at 0.09 mc. per mmole was received from Tracerlab, Inc., Boston, Massachusetts, under authorization from the Atomic Energy Commission.

The recrystallized product had the following properties: m.p., 304–307° (reported (18), 305°); sulfur content, 25.40 per cent (theoretical, 25.62 per cent). The R_F values and ninhydrin chromogenicity with paper chromatography were comparable to those of pure unlabeled taurine. The specific activity was 0.6 μc. per mg. The first recrystallization gave a loss of 2.8 per cent of the specific activity.

The taurocholate-S^{35} was biosynthesized as follows: 12 mg. of taurine-S^{35} (0.6 μc. per mg.) were intubated into the duodenum of each of five bile-

TABLE I

Per Cent Composition of Purified Diets

	Group II	Group IV	Group V
α-Protein*	10.0	0.0	10.0
Casein (vitamin-free)	0.0	30.0	0.0
Cholesterol†	5.0	5.0	0.0
Corn oil	15.0	15.0	8.0
Sucrose	65.6	45.6	77.6
Inositol	0.1	0.1	0.1
Choline	0.2	0.2	0.2
p-Aminobenzoic acid	0.1	0.1	0.1
Salt mixture (30)	4.0	4.0	4.0

Each kilo of diet supplied α-tocopherol 100 mg., menadione 5 mg., vitamin A 12,500 U. S. P. units, vitamin D 1800 U. S. P. units, thiamine 5 mg., riboflavin 5 mg., pyridoxine 2.5 mg., calcium pantothenate 50 mg., niacin 80 mg., folic acid 0.25 mg., and biotin 0.20 mg.

Group I received Purina animal chow. Group III, identical with that of Group II except for the presence of 0.6 per cent DL-methionine added.

* α-Protein is a derivative of soy beans prepared by The Glidden Company, Chicago, Illinois, and was furnished for this work through the courtesy of Mr. W. M. Bain.

† Crystalline cholesterol was generously supplied by Armour and Company, Chicago, Illinois.

cannulated rats (350 gm.), and the bile was collected for 24 hours. The mucin was precipitated with 4 volumes of absolute alcohol and removed. The filtrate was taken to dryness *in vacuo* and redissolved in 10 cc. of water. The pH was adjusted to 1.0, and a butanol-water partition was performed according to the method of Bergström *et al.* (12). This partition left free taurine in the water phases and bile acids in the butanol phases. The combined butanol phases were taken to dryness *in vacuo* and redissolved in 20 cc. of water plus 1 drop of 1 N sodium hydroxide. The 4 cc. aliquots used as a single animal dose contained 0.5 μc. of S^{35}. A similar preparation of non-labeled taurocholate was prepared with added free taurine-S^{35} as a control treatment.

The labeled taurine was administered in aqueous solution into the duodenostomy at zero time in the amount of 9 mg. (0.6 μc. per mg.) per kilo of body weight. Preliminary studies with normal animals indicated that this dose results in a dilution of administered taurine by intrinsic taurine of 1:100 in a 24 hour bile collection. The labeled taurocholate was supplied through the duodenostomy in a volume of 4 cc., given over a period of 4 hours as a continuous drip.

Method of Radioassay—Aliquots of bile, urine, and fecal extracts were digested by the method of Benedict (19) for the sulfur radioassays. In some instances the ethanol-soluble fractions (protein-free) and butanol-soluble fractions (taurine-free) of bile were similarly digested. Solid tissues were hydrolyzed with a hydrochloric acid-formic acid mixture (20) and neutralized to pH 7.0 before addition of the oxidizing mixture. The use of 1 cc. of Benedict's solution for 20 mg. of organic matter gave a smooth oxidation of 98 per cent or more of added taurine to sulfate in preliminary trials. A sufficient known amount of sodium sulfate was added to bring the resulting quantity of barium sulfate above 50 mg. or into the range of infinite thickness for the sample geometry used. The barium sulfate samples were filtered and washed on Eaton-Dikeman No. 613 paper by means of a stainless steel filtration device. The samples were weighed and then counted with a thin window Geiger-Müller tube to a probable error of 2 per cent. The counts were corrected for decay and self-absorption, and the results were expressed in terms of per cent of the total dose administered and, in some instances, as specific activities of $BaSO_4$.

Bile Acid Assay—Bile acids were assayed by a spectrophotometric method to be described in detail elsewhere. The method consisted essentially of a cholic acid determination by the modified Pettenkofer reaction of Irvin, Johnston, and Kopala (21) and a determination of dihydroxycholanic acids by a measurement of the Minibeck reaction (22) at 310 mμ (where the absorption spectrum of dihydroxycholanic acids shows a maximum) on material which had previously been cleared of pigment, cholesterol, and fatty acids. The measurement was corrected for the cholic acid contribution. The results were expressed as free bile acids without reference to the state of conjugation.

Taurine-Glycine Ratios—In some experiments the quantities of glycine and taurine in the alkaline hydrolysate of alcohol-soluble portions of bile and in unhydrolyzed ethanolic filtrates were estimated. The alcoholic filtrates were made 2.5 N with sodium hydroxide and autoclaved at 125° for 3 hours. The hydrolysates were adjusted to pH 1.0 with hydrochloric acid; the precipitated bile acids were filtered and the filtrates adjusted to pH 7.0. Aliquots of these filtrates were then subjected to one-dimensional

ascending paper chromatography with acetone-water-urea (23). Ninhydrin colors at the taurine and glycine loci were estimated spectrophotometrically by the method of Naftalin (24), and the taurine-glycine ratios were determined.

RESULTS AND DISCUSSION

Excretion of S^{35} and Bile Acids—A typical excretion curve for neutral S^{35} by a normal rat following dosage with taurine-S^{35} through a duodenostomy is presented in Fig. 1. Both the total and inorganic sulfate of the urine were essentially free of S^{35}. The maximal rate of excretion in urine

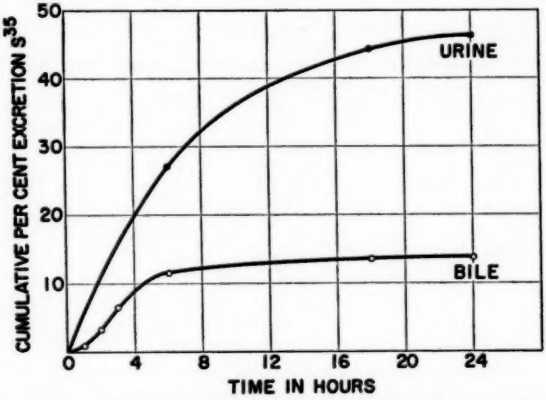


FIG. 1. The excretion of S^{35} following administration of taurine-S^{35} (3 mg. and 1.8 μc.).

and bile occurred during the first 6 hours after administration of the test dose, and the excretion had virtually ceased at the end of 24 hours. At that time, however, only 60 per cent of the dose had been accounted for in these excretions.

Effect of Preliminary Dietary Treatment on Excretion of S^{35}—The effects of the preliminary dietary treatment on the animal's disposition of administered dose of taurine-S^{35} are best considered in relation to the chow-fed animals. The five chow-fed rats (Group I) excreted an average of 62.8 per cent of the sulfur label in a period of 24 hours (Table II). About one-fourth of the excreted S^{35} was found in the bile and about three-fourths in the urine. Less than 2 per cent was present in the feces. It is clear that administered taurine is efficiently absorbed from the intestine and largely excreted in the urine and bile.

In investigating the nature of the compounds in which S^{35} existed in

bile, it was found that the bile from rats in all dietary groups contained essentially equal total activity in equivalent amounts of whole bile, ethanol-soluble (protein-free) portions, and butanol-soluble (taurine- and inorganic sulfate-free) portions of bile. The S^{35} thus was present in the bile as taurine conjugated with bile acids.

The six rats of Group II, fed diets low in organic sulfur and high in cholesterol and fat, excreted a total of 23.7 per cent of the S^{35} administered in 24 hours. This lower recovery was accounted for by the great reduction of urinary labeled sulfur ($t = 3.26$, $p = 0.01$) compared to that of chow-fed controls. The quantity of biliary S^{35} excreted was little different from that of Group I.

TABLE II

Excretion of S^{35} in 24 Hours after Treatment with Taurine-S^{35} of Rats Maintained on Different Diets

Group No.	No. of animals	Specific activity of bile*	Per cent of total dose†			
			Bile	Urine (neutral sulfur)	Feces‡	Total
I	5	157	16.7 ± 4.6	44.8 ± 11.6	1.3	62.8
II	6	422	14.6 ± 3.3	6.4 ± 2.7	2.7	23.7
III	6	94	6.2 ± 1.7	42.6 ± 8.0	2.3	51.1
IV	4	196	6.8 ± 2.7	35.0 ± 12.1	1.6	43.4
V	5	532	11.9 ± 3.5	18.8 ± 6.5	2.9	33.6

* Specific activity expressed as counts per minute per mg. of $BaSO_4$ recovered from bile. Counts corrected for decay and self-absorption.

† Mean ± standard error of the mean.

‡ Two animals of each group were used for the fecal determination of radiosulfur.

The rats of Group III differed from those of Group II only by the presence of 0.6 per cent DL-methionine in the preparatory diet. These animals excreted an average of 51.1 per cent of the S^{35} dose. The biliary excretion of S^{35} was lower than that of Group I ($t = 2.16$, $p = 0.06$) or Group II ($t = 2.26$, $p = 0.05$), although the urinary excretion of S^{35} was similar to that of the controls of Group I.

Group IV, prepared with a high protein, high fat, and cholesterol diet, excreted an average of 43.4 per cent of the administered taurine. The lower total excretion of S^{35} was accounted for by a lowered biliary excretion compared to Group I ($t = 1.90$, $p = 0.09$).

Group V was fed a diet deficient in organic sulfur, but with intermediate fat levels and without added cholesterol. The reduced S^{35} excretion (33.6 per cent) was accounted for by the lower excretion in urine (18.8 per cent) below that of Group I ($t = 1.97$, $p = 0.08$), Group III, or Group IV. The biliary S^{35} was not significantly lower than that of Group I or II.

These observations of the variation in biliary excretion of taurine-S^{35} could be explained in several ways. The variations in excretion of S^{35} in the bile could be interpreted as a reflection of the quantity of bile acid synthesized or as a variable dilution of labeled taurine in intrinsic taurine available for conjugation. A comparison of the recovered biliary sulfur in the different diet groups shows that animals fed diets low in organic sulfur had markedly higher specific activities (Table II). Thus, dilution of the administered taurine was an important cause of the differences observed. An examination of the arbitrary ratio relating the per cent of the dose of radiosulfur excreted in the bile in 24 hours to the 24 hour excretion of bile

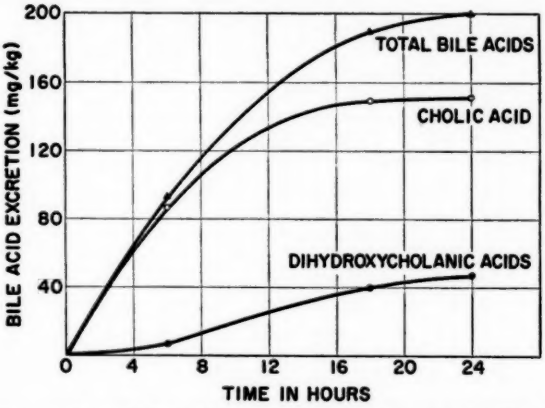


FIG. 2. The excretion of bile acids by a chow-fed rat prepared with a bile fistula

acids in mg. also confirmed this dilution of the administered taurine (Table III).

Excretion of Bile Acids—Fig. 2 illustrates a typical excretion pattern for cholic acid, dihydroxycholanic acids, and total bile acids in the chow-fed rat. It was noted that the initial bile excreted contained cholic acid almost exclusively, whereas later in the collection period dihydroxycholanic acids (presumably chenodeoxycholic, Bergström *et al.* (12)) were found.

Effect of Dietary Preparation on Bile Acid Excretion—The most striking observation made on the pattern of bile acid excretion is the decidedly lower levels of excretion by animals on synthetic diets (Table III). The rats in Group I (fed Purina chow) excreted about 4 times as much bile acid, on a body weight basis, as did those in Group V. However, there were no significant differences among the animals on the various synthetic diets. The data also indicate that in the rats with reduced levels of bile acid excretion a larger part of the excreted bile was in the form of dihy-

droxycholanic acids. This information, together with the pattern of bile acid excretion with time (Fig. 2), indicated that under normal conditions of diet and reabsorption of bile in the rat the most prevalent bile acid is cholic acid, but that under our particular conditions the bile acids of lower oxidation states become increasingly prominent in the bile.

Tissue Levels of S^{35}—Since the maximal radiosulfur recovered in 24 hours in the bile, urine, and feces of any rat was 85 per cent, and, since at that time the excretion rate had become very low, it was of interest to determine the location of the remaining sulfur in the perfused animal. There seemed little doubt that some of the radiosulfur remained in tissues with a rather long biological half life. The sulfur-deficient animals from Groups II and V had the highest tissue retention (Table IV) of sulfur at 24 hours. Al-

TABLE III

24 Hour Excretion of Bile Acids by Rats Maintained on Several Diets (Mg. per Kilo of Body Weight)

Group No.	Cholic acid	Dihydroxycholanic acid	Total bile acid	Per cent S^{35} in 24 hrs. per mg. bile acids per kilo body weight
I	123.6	37.2	160.8 ± 41.6*	0.075
II	47.7	29.8	77.4 ± 19.4	0.220
III	25.5	31.1	56.7 ± 22.4	0.125
IV	35.4	40.3	75.8 ± 22.4	0.086
V	24.8	18.8	43.6 ± 10.8	0.295

* Mean ± standard error of the mean (for the same number of animals as in Table II).

though all tissues contained some radiosulfur, the musculoskeletal system retained much the greater total amount. Specific activities were highest for kidney and pooled visceral organs in every group.

It was surprising, in view of the findings of Lewis and Lewis (25), *i.e.* that taurine could not substitute for cystine in growing rats, to find this high tissue fixation of labeled sulfur. It was concluded that animals maintained on diets containing inadequate quantities of organic sulfur were able to utilize taurine sulfur in the synthesis of other organic sulfur compounds. Dziewiatkowski has shown that the normal rat can utilize sulfate sulfur in the synthesis of cystine (26) and chondroitin sulfates (27). This tissue utilization of taurine sulfur was also reflected in the experience of one of us (G. V. M.) that feeding taurine to the *Cebus* monkey deficient in organic sulfur resulted in a small but significant growth stimulus. The simultaneous high biliary excretion of taurine-S^{35} and tissue fixation of S^{35} in Group II and Group V suggested that loss of bile sulfur was an obliga-

tory process in the rat and that the bile loss aggravated deficiency of dietary organic sulfur.

Taurine-Glycine Ratios in Bile—Paper chromatographic determinations indicated that in the rat the majority of biliary bile acids was conjugated with taurine, as claimed by Bergström *et al.* (12). The taurine-glycine ratios (corrected for free amino acids in bile) during a 24 hour collection on a rat fed Purina chow was 27.0.

TABLE IV

Distribution of S^{35} in Rat Tissues at 24 Hours after Administration of Taurine-S^{35} and Bile Collection

The results are in per cent of the total dose (3 mg. (0.6 μc. per mg.) taurine-S^{35} per kilo of body weight).

	Diet I, Rat 19	Diet II, Rat 9	Diet III, Rat 8	Diet V, Rat 2
Liver	0.73	1.87	0.80	1.98
Kidneys	0.84	2.31	1.03	2.39
Other viscera	3.93	9.61	4.33	8.90
Blood*	7.64	2.94	3.82	2.51
Skin and hair	6.01	15.60	7.16	14.98
Musculoskeletal	13.01	34.11	26.33	30.05
Total	32.16	66.44	43.47	60.81
Bile (24 hrs.)	14.62	17.20	5.14	18.00
Urine	49.19	4.28	50.65	8.54
Feces + intestinal washing	2.30	3.48	2.65	4.01
Total	66.11	24.96	58.44	30.55
" recovery	98.27	91.40	101.91	91.36

* Total blood radiosulfur calculated on the assumption that blood volume equals 10 per cent of the body weight.

Metabolism of Taurocholate-S^{35}—The comparison of excretion in bile and urine (Table V) of S^{35} following intubation of taurocholate-S^{35} and of unlabeled taurocholate plus taurine-S^{35} showed that there was a much higher biliary excretion of radiosulfur, 69.2 per cent (43.2 to 92.7), and lower urinary excretion of S^{35}, 8.5 per cent (2.1 to 18.7), in the rats fed taurocholate-S^{35}. This indicated that most of the bile acid excreted was reabsorbed from the intestine and reexcreted without hydrolysis of taurocholate. No radiosulfur was found in the lymph of a thoracic duct-cannulated animal. Since the bile acids were shown to be absorbed in the conjugated form, this observation confirms the claim of Josephson and Rydin (28)

that the bile acids are not absorbed by the lymphatics. Thus our findings are consistent with the classical hypothesis of Schiff (29) that there is an enterohepatic circulation of conjugated bile acids.

The findings of Mosbach[3] that only free bile acids were found in the feces of dogs could be explained by assuming either that the reabsorption of conjugated bile acids was complete or that the conjugated bile acids escaping reabsorption were completely hydrolyzed in their passage through the remainder of the gastrointestinal tract.

TABLE V

Excretion of S^{35} during 24 Hours Following Administration of Taurocholate-S^{35} and Unlabeled Taurocholate Plus Taurine-S^{35} to Rats

Dosage	Per cent total dose		
	Bile	Urine	Feces
Taurocholate-S^{35} (0.5 μc.); 4 rats	69.2 (43.2–92.7)	8.5 (2.1–18.7)	10.8
Unlabeled taurocholate + taurine-S^{35} (0.9 μc.); 2 rats	12.2 (9.8–14.7)	46.1 (41.5–50.7)	4.4

SUMMARY

1. The disposition of taurine-S^{35}, supplied by duodenostomy, was studied in rats prepared for 45 to 60 days on dietary regimens containing various levels of organic sulfur, fat, and cholesterol. Rats fed the chow diet excreted 16.7 per cent of the radiosulfur in the bile and 44.8 per cent in the urine. Those fed diets low in organic sulfur excreted comparable amounts of radiosulfur in the bile, but much lower quantities in the urine.

2. Evidence was presented which indicates that the majority of the biliary radiosulfur was conjugated with bile acids.

3. Rats fed diets low in organic sulfur had higher specific activities for biliary sulfur. This was offered as evidence that the biliary excretion of radiosulfur reflects the dilution of extrinsic (taurine-S^{35}) in intrinsic taurine.

4. Studies of the 24 hour excretion of bile acids by bile fistula indicate that there is a progressive reduction with time in the quantity of bile acid excreted by the rat and a shift toward excretion of dihydroxycholanic acids. All of the groups fed synthetic diets, which were low in organic sulfur or high in fat and cholesterol, excreted lesser amounts of bile acid than did rats fed chow.

5. Tissue levels of S^{35} at 24 hours after administration of taurine-S^{35} were high. Rats fed diets low in organic sulfur retained larger amounts

[3] Mosbach, E. H., personal communication.

of S^{35}. The greater part of the activity was in the musculoskeletal system in all animals. Higher specific activities were found in kidneys and pooled organs.

6. Biosynthetic taurocholate-S^{35} was largely reabsorbed from the intestine and reexcreted in the bile (mean = 69.2 per cent). Thoracic duct lymph was free of radiosulfur. This was offered as proof of the Schiff hypothesis of enterohepatic circulation of conjugated bile acids.

The technical assistance of Miss Ann McNally was essential for this work.

BIBLIOGRAPHY

1. Mann, G. V., Andrus, S. B., McNally, A., and Stare, F. J., *J. Exp. Med.*, **98**, 195 (1953).
2. Fillios, L. C., and Mann, G. V., *Metabolism*, **3**, 16 (1954).
3. Tarver, H., and Schmidt, C. L. A., *J. Biol. Chem.*, **146**, 69 (1942).
4. Awapara, J., *Nature*, **165**, 76 (1950).
5. Boström, H., and Aqvist, S., *Acta chem. Scand.*, **6**, 1557 (1952).
6. Binet, L., Pettavel, J., and Wellers, G., *Compt. rend. Acad.*, **232**, 1461 (1951).
7. Haslewood, G. A. D., and Wootton, V., *Biochem. J.*, **47**, 584 (1950).
8. Bloch, K., Berg, B. N., and Rittenberg, D., *J. Biol. Chem.*, **149**, 511 (1943).
9. Zabin, I., and Barker, W. F., *J. Biol. Chem.*, **205**, 633 (1953).
10. Byers, S. O., Friedman, M., Biggs, M. W., and Gunning, B., *J. Exp. Med.*, **97**, 511 (1953).
11. Bergström, S., and Norman, A., *Proc. Soc. Exp. Biol. and Med.*, **83**, 71 (1953).
12. Bergström, S., Dalquist, A., and Lundquist, V., *Kungl. Fysiograf. Sällskap. Lund, Förhandl.*, **23**, 1 (1953).
13. Foster, M. G., Hooper, C. W., and Whipple, G. H., *J. Biol. Chem.*, **38**, 379, 421 (1919).
14. Hawkins, W. B., Hanson, P. C., Coon, R. W., and Terry, R., *J. Exp. Med.*, **90**, 461 (1949).
15. White, A., *J. Biol. Chem.*, **112**, 503 (1935–36).
16. Bollman, J. L., Cain, J. C., and Grindlay, J. H., *J. Lab. and Clin. Med.*, **33**, 1349 (1948).
17. Cortese, F., *Org. Syntheses*, **13**, 13 (1938).
18. Cortese, F., *Org. Syntheses*, **13**, 77 (1938).
19. Benedict, S. R., *J. Biol. Chem.*, **6**, 363 (1909).
20. Bolling, D., and Block, R. J., *Arch. Biochem.*, **2**, 93 (1943).
21. Irvin, J. L., Johnston, C. G., and Kopala, J., *J. Biol. Chem.*, **153**, 439 (1944).
22. Minibeck, H., *Biochem. Z.*, **297**, 29 (1938).
23. Bentley, H. R., and Whitehead, J. K., *Biochem. J.*, **46**, 341 (1950).
24. Naftalin, L., *Nature*, **161**, 763 (1948).
25. Lewis, G. T., and Lewis, H. B., *J. Biol. Chem.*, **69**, 589 (1926).
26. Dziewiatkowski, D. D., *J. Biol. Chem.*, **207**, 181 (1954).
27. Dziewiatkowski, D. D., *J. Exp. Med.*, **98**, 119 (1953).
28. Josephson, B., and Rydin, A., *Biochem. J.*, **30**, 2224 (1936).
29. Schiff, M., *Arch. ges. Physiol.*, **3**, 598 (1870).
30. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **138**, 459 (1941).

CARBOHYDRATE METABOLISM BY PSEUDOMONAS FLUORESCENS

III. PURIFICATION AND PROPERTIES OF A 6-PHOSPHOGLUCONATE DEHYDRASE*

By R. KOVACHEVICH and W. A. WOOD

(From the Laboratory of Bacteriology, Department of Dairy Science, University of Illinois, Urbana, Illinois)

(Received for publication, August 19, 1954)

Extracts of *Pseudomonas fluorescens* catalyze the oxidation of glucose and gluconate to 2-ketogluconate without the involvement of substrate phosphorylation (1). Such extracts also carry out a rapid and essentially complete oxidation of glucose-6-phosphate and 6-phosphogluconate (6-PG) (2).

Entner and Doudoroff (3) have demonstrated the presence of enzymes in *Pseudomonas saccharophila* which degrade 6-PG to pyruvate and triose phosphate by reactions not involving ribose-5-phosphate or carbon dioxide. Carbon atoms 1, 2, and 3 of 6-PG form pyruvate with the carboxyl group of pyruvate arising from carbon atom 1; triose phosphate arises from carbon atoms 4, 5, and 6. A reaction mechanism involving the formation of 2-keto-3-deoxy-6-phosphogluconate (KDPG), followed by scission between carbon atoms 3 and 4 to form pyruvate and triose phosphate, was postulated by Entner and Doudoroff (3). After incubating suitably treated enzyme preparations with 6-PG, the KDPG was recovered as the crystalline trisodium salt and characterized (4).

Wood and Schwerdt (2) have reported that cell-free extracts of *P. fluorescens* produce pyruvate and triose phosphate from 6-PG by a similar system at approximately 10 times the rate of 6-PG oxidation by 6-PG dehydrogenase. In order to establish the steps in this new pathway and the cofactors involved, separation and purification of the enzymes involved have been undertaken. Fractionation of cell-free extracts of *P. fluorescens* with ammonium sulfate and acetone has yielded a 27-fold purified enzyme preparation which does not produce pyruvate and triose phosphate, but which converts 6-phosphogluconate to a carbonyl-containing phosphate ester having the characteristics of authentic 2-keto-3-deoxy-6-phosphogluconate. Ferrous ions and glutathione (GSH) are required for activity.

* This research was supported in part by a grant-in-aid from the National Science Foundation. A preliminary account was presented at the annual meeting of the Society of American Bacteriologists, Pittsburgh, Pennsylvania, May, 1954.

Methods

Bacteriological—P. fluorescens, strain A3.12, was grown at room temperature in a mineral medium described previously (5). One or more carboys containing 14 liters of medium were each inoculated with 1 liter of a 16 hour culture grown in 1 per cent yeast extract, 1 per cent tryptone, 0.5 per cent K_2HPO_4, and 0.5 per cent glucose. Vigorous aeration was attained by an arrangement which stirred at 1725 r.p.m. and forced 1 to 2 volumes of air per minute through the culture. After 7 hours the cells were harvested in a Sharples centrifuge, washed, and dried either *in vacuo* or with acetone. 2 to 2.5 gm. dry weight of cells per liter of medium were obtained.

Chemical—6-Phosphogluconate was prepared from crystalline barium glucose-6-phosphate heptahydrate (6) by a modification of the procedure of Robison and King (7) in which the bromine oxidation was carried out in the absence of barium ions. 1-C^{14}-6-Phosphogluconate was prepared by Dr. B. L. Horecker (8). Sodium glutathione (NaGSH) was obtained from the Schwarz Laboratories, Inc. The end-product, presumed to be KDPG, was prepared from 6-PG by the action of 6-PG dehydrase and recovered from the reaction mixture as the monobarium salt by alcohol precipitation. Crystalline Na_3-2-keto-3-deoxy-6-phosphogluconate·$2H_2O$ was donated by Dr. M. Doudoroff. Triphosphopyridine nucleotide, 80 per cent pure, was obtained from the Sigma Chemical Company. DL-Glyceraldehyde-3-phosphate (G-3-P) was prepared from DL-glyceraldehyde-1-bromide-3-phosphoric acid (dimeric) obtained from the Concord Laboratories by neutralization with sodium hydroxide.

Determinations—Pyruvate was determined either by the direct or by the double extraction methods of Friedemann and Haugen (9). In the absence of both hydrazine and the alkali pretreatment which are employed in aldolase determinations by Sibley and Lehninger (10), glyceraldehyde-3-phosphate did not give a color. KDPG formed a 2,4-dinitrophenylhydrazone which was not soluble in polar solvents. In the presence of alkali the color produced by the unextracted 2,4-dinitrophenylhydrazone faded to nil in less than 5 minutes. Thus the intermediate phosphate ester and glyceraldehyde-3-phosphate did not interfere with the pyruvate determinations. Pyruvate also was estimated in the presence of lactic dehydrogenase and reduced diphosphopyridine nucleotide (DPNH) from the decrease in optical density at 340 mμ. 6-Phosphogluconate was determined spectrophotometrically by the method of Horecker and Smyrniotis (11) with purified yeast 6-PG dehydrogenase. KDPG was determined by ceric sulfate degradation (12) or by conversion to pyruvate by KDPG aldolase (13), followed by spectrophotometric or colorimetric pyruvate determinations. Carbon[14] concentrations were determined as $BaC^{14}O_3$, as described

by Van Slyke *et al.* (14). Protein was determined by the method of Lowry *et al.* (15).

Enzyme Assay—Routine measurements of the reaction rate were made in the presence of excess KDPG aldolase by following pyruvate formation. Under these conditions the amount of 6-PG dehydrase governed the rate of pyruvate formation. The 1 ml. reaction mixtures contained 200 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 7.65, 3 μmoles of NaGSH, 6 μmoles of $FeSO_4$, 7 μmoles of Na 6-PG, and 2 to 4 units of KDPG aldolase. The order of additions was found to be important. After the enzymes were added to the buffer and water, NaGSH was added next, and this was followed by the $FeSO_4$ within approximately 3 minutes. The reaction mixture then was equilibrated at 37° for 10 minutes before the addition of 6-PG. After a 10 minute reaction period, 1 ml. of 2,4-dinitrophenylhydrazine reagent was added and the amount of pyruvate determined. Under these conditions the rate of pyruvate formation was linear with time over a 20 minute period and was essentially proportional to enzyme concentration over a wide range (0.25 to 1.25 units, Fig. 1). This method was applicable to the assay of crude preparations in either the presence or absence of sodium arsenite. A unit of 6-PG dehydrase was arbitrarily defined as the amount of enzyme which formed 1 μmole of pyruvate in 10 minutes under the above conditions. Specific activity is expressed as units per mg. of protein.

In some instances 6-PG dehydrase activity was followed by the rate of 6-PG disappearance or by the rate of KDPG formation. For such assays, aliquots of the reaction mixtures were removed, the reaction was stopped by adding trichloroacetic acid or by heating, and the 6-PG concentration was determined with 6-PG dehydrogenase or the KDPG concentration spectrophotometrically with KDPG aldolase, lactic dehydrogenase, and DPNH (13).

Results

Enzyme Fractionation—6-PG dehydrase was prepared from either vacuum- or acetone-dried cells by approximately the same procedure. The acetone-dried cells, however, contained approximately twice as much 6-PG dehydrase as KDPG aldolase, whereas vacuum-dried cells contained about 6 times as much KDPG aldolase as 6-PG dehydrase. When it was desired to prepare both enzymes, the vacuum-dried cells were used.

Extraction—20 gm. of dried cells were suspended in 400 ml. of 0.1 M phosphate buffer, pH 7, and subjected to sonic vibration at 10 kc. for 10 to 15 minutes. Virtually all of the activity was present in the soluble protein fraction.

Ammonium Sulfate Precipitation—In all of the ammonium sulfate pre-

cipitations, a solution saturated at 1° and neutralized with NH_4OH to pH 7.3 at 2° was used. To each 100 ml. of extract, 49 ml. (0.25 saturation) of saturated ammonium sulfate were added. The cellular débris and particles were removed by centrifugation at approximately 10,000 × *g* and discarded. 84 ml. of saturated ammonium sulfate were added to each 100 ml. of the supernatant solution (0.5 saturation). The protein precipitate was separated by centrifugation at 10,000 × *g* and dissolved in 100 ml. of 0.01 M phosphate buffer, pH 7.0.

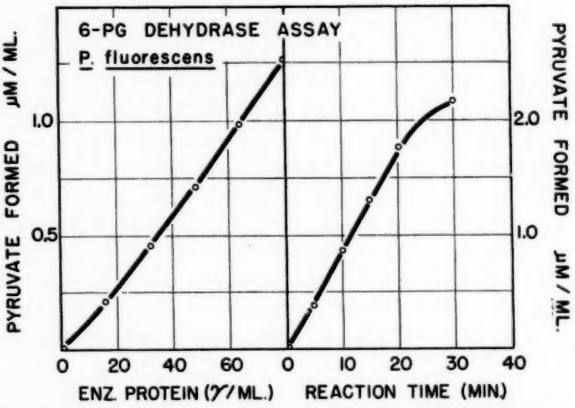


FIG. 1. Pyruvate formation as a function of time and 6-PG dehydrase concentration. The reaction mixture contained 200 μmoles of tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 7.65, 2 units of KDPG aldolase (40 to 50 per cent $(NH_4)_2SO_4$ fraction), 3 μmoles of NaGSH, 6 μmoles of $FeSO_4$, 7 μmoles of Na-6-PG, 6-PG dehydrase, and water to 1 ml. In the left-hand portion of the graph the amount of 6-PG dehydrase (40 units per mg. of protein) was as indicated, and the incubation time at 37° was 10 minutes. In the right-hand graph, 0.42 unit of enzyme was used and the incubation time was as indicated.

Protamine Precipitation—To each 100 ml. of ammonium sulfate fraction, 20 to 25 ml. of protamine sulfate (20 mg. per ml. adjusted to pH 5.0 with dilute acetic acid) were added. The precipitate which formed immediately was removed by centrifugation at 18,000 × *g*. Potassium phosphate buffer (1 M) was added to the clear supernatant solution to bring the phosphate concentration to 0.1 M. The ratio of optical density at 280 and 260 mμ was approximately 0.9. At this stage, the enzyme preparation was stored overnight at −14° with only a slight loss in activity.

Ammonium Sulfate Precipitation—113 ml. of saturated ammonium sulfate per 100 ml. of enzyme (0.40 saturation) were added to the protamine supernatant solution, and the precipitate was collected by centrifugation

and redissolved in 37 ml. of 0.1 M phosphate buffer, pH 7.0. This precipitate contained most of the 6-PG dehydrase but was highly contaminated with KDPG aldolase. The addition of more saturated ammonium sulfate to the supernatant solution (27 ml. per 100 ml. of solution (0.5 saturation)) yielded a precipitate which contained most of the KDPG aldolase activity.

Acetone Fractionation—1 volume of acetone (−10°) was added to the 0.4 ammonium sulfate fraction. During the addition of acetone, the temperature rose to 5–7°. The precipitate was removed by centrifugation at 23,000 × *g* and discarded. During centrifugation the temperature decreased to 2–3°. An additional 0.5 volume of acetone was added, and the precipitate was collected by centrifugation and dissolved in 33 ml. of 0.1 M phosphate buffer, pH 7.0. A second acetone precipitation was carried out in the same manner. The material which precipitated between 1.25 and 1.75 volumes of acetone was redissolved in 14 ml. of 0.1 M phosphate buffer, pH 7.5. The 1.25 to 1.75 acetone fractionation was repeated and the precipitate redissolved in 7 ml. of 0.01 M phosphate buffer.

By this procedure the 6-PG dehydrase was obtained 27-fold purified and free of the KDPG aldolase (Table I). The dehydrase activity deteriorated slowly at −14°, but the preparation was usable over a 2 week period.

Properties of 6-PG Dehydrase—The rate of 6-PG utilization was initially more rapid than the rate of KDPG formation, at 10 minutes the difference being about 1.5 μM (Fig. 2). After 40 minutes the 6-PG was completely utilized and an equivalent amount of KDPG was formed. The addition of more enzyme after 1 hour was without effect, except that a gradual disappearance of KDPG was noted. Pyruvate was not formed during the reaction.

The ability of 6-PG dehydrase to catalyze the reverse reaction (hydrate KDPG) was tested in the presence of 6-PG dehydrogenase and triphosphopyridine nucleotide with KDPG as the substrate. TPN reduction was not observed. Thus the equilibrium of the dehydration reaction appears to be completely toward KDPG formation.

Activation by Glutathione and Ferrous Ions—6-PG dehydrase was inactive as measured by either 6-PG disappearance or by pyruvate formation, unless both GSH and ferrous ions were present (Table II). In the absence of KDPG aldolase, 3.2 μmoles of 6-PG disappeared when GSH and Fe^{++} ions were present. When either Fe^{++} or GSH was added singly, no activity was evident. In the presence of KDPG aldolase, GSH, and Fe^{++}, the utilization of 6-PG (2.6 μM) was accompanied by the formation of an equivalent amount of pyruvate. The omission of either GSH or Fe^{++} greatly diminished the reaction rate. Since the disappearance of 6-PG was dependent upon GSH and Fe^{++} and was not dependent upon KDPG aldolase, it is evident that these activators are required for 6-PG dehydrase

action. It later was shown that KDPG aldolase activity was not dependent upon these activators (13).

A rapid increase in activity was obtained by incubating the enzyme with GSH for 2 to 3 minutes before adding the Fe^{++}. Increasing the time

TABLE I

Purification of 6-Phosphogluconate Dehydrase from Vacuum-Dried P. fluorescens

Step No.		Activity	Specific activity, units per mg. of protein	6-PG dehydrase / KDPG aldolase
		units		
1	Sonic extract	10,430	1.6	0.49
2	0.25–0.50 saturated $(NH_4)_2SO_4$ ppt.	7,040	2.1	0.27
3	Protamine supernatant	3,820	1.2	0.22
4	0–0.4 $(NH_4)_2SO_4$ ppt.	4,720	2.3	0.40
5	1st acetone ppt. (1–1.5 volumes)	2,120	18	1.8
6	2nd " " (1.25–1.75 volumes)	1,430	36	∞
7	3rd " " (1.25–1.75 ")	710	42	∞

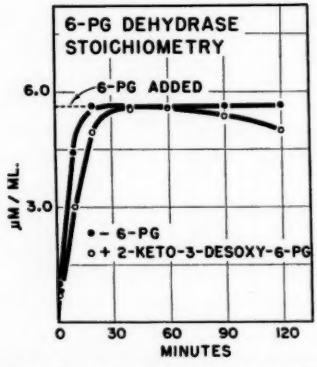


FIG. 2. The conversion of 6-PG to KDPG by 6-PG dehydrase. The conditions were as described in Fig. 1, except that 3 units of 6-PG dehydrase and 5.6 μmoles of 6-PG were used. Aliquots were removed at the intervals indicated, heated in boiling water for 2 minutes, and a suitable dilution assayed for 6-PG and KDPG.

between these additions decreased the activity. After incubation of enzyme and GSH in the absence of Fe^{++} for 10 minutes, no activity was observed. When Fe^{++} was added before GSH, only slight activation was observed.

In addition to ferrous ions, manganous and magnesium ions were par-

tially effective activators. Calcium, zinc, cobaltous, and ferric ions (5×10^{-3} M) were inactive. The activity of 6-PG dehydrase as a function of the Fe^{++}, Mn^{++}, and Mg^{++} concentration is shown in Fig. 3. The greatest activation was obtained with 4×10^{-3} M Fe^{++}. Mn^{++} was 70 per cent as effective at an optimal concentration of 10^{-4} M. The activity with Mg^{++} (10^{-2} M) was 45 per cent of that obtained with Fe^{++}. The failure of small amounts of Fe^{++} (5×10^{-4} M or less) to activate the 6-PG dehydrase was attributed either to an oxidation to Fe^{+++} or to the binding of Fe^{++} by sulfhydryl groups and phosphate ions. This phenomenon was not displayed by Mn^{++} or Mg^{++}. The addition of combinations of Fe^{++} with

TABLE II

Activation of 6-Phosphogluconate Dehydrase by Ferrous Ions and Glutathione

Conditions as described in Fig. 1, except that NaGSH (3 μmoles per ml.), 6-PG dehydrase (1.6 units), and KDPG aldolase (2 units) were added as indicated. The incubation time was 20 minutes.

Additions		− Δ 6-PG	+ Δ pyruvate
Enzyme	Activators		
6-PG dehydrase		Trace	Trace
" "	GSH	0	0
" "	Fe^{++}	0	0
" "	" GSH	3.2	0.1
KDPG aldolase	" "	0	0
6-PG dehydrase + KDPG aldolase		0	0.1
" " + " "	GSH	0	0.1
" " + " "	Fe^{++}	0	0.4
" " + " "	" GSH	2.6	2.6

Mn^{++} or Mg^{++} resulted in activation which was intermediate between the optimal values of the individual ions.

The effect of thiol concentration upon dehydrase activity is shown in Fig. 4. The greatest activation was obtained with GSH. Cysteine was approximately 64 per cent as effective at 7.5×10^{-3} M concentration. Thioglycolate gave a slight activation at low concentrations, whereas ascorbate and oxidized glutathione were ineffective. 7.5 to 10 μmoles of GSH were required for maximal activity. However, many other 6-PG dehydrase preparations were maximally active in the presence of 3 to 6 μmoles of GSH.

Substrate—The effect of substrate concentration upon the reaction velocity is shown in Fig. 5. The maximal rate was attained with 4 μmoles per ml. and the amount necessary for the one-half maximal rate was approxi-

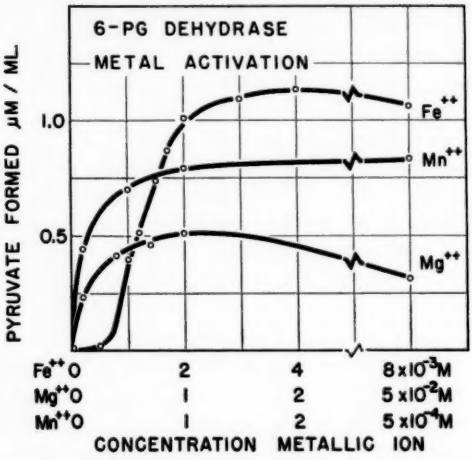


FIG 3. The effect of divalent ion concentration upon 6-PG dehydrase activity. The reaction mixture was the same as in Fig. 1, except that 0.8 unit of 6-PG dehydrase was added, and the concentrations of $FeSO_4$, $MnCl_2$, and $MgSO_4$ were as indicated. The incubation time was 10 minutes.

mately 0.5 µmole per ml. The Michaelis constant obtained from plots according to Lineweaver and Burk (16) (Fig. 5, inset) was $K_m = 6 \times 10^{-4}$ mole per liter.

pH Optimum—The effect of pH upon the reaction rate is shown in Fig. 6. The activity was determined by measuring the rate of 6-PG disappear-

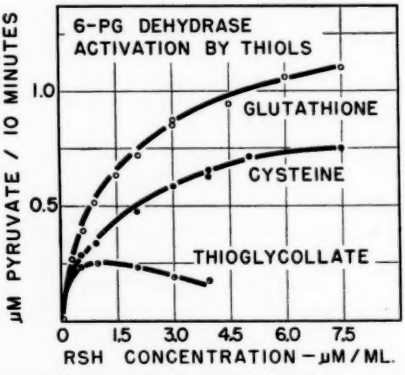


FIG. 4. The effect of thiol concentration on 6-PG dehydrase. The conditions were as in Fig. 1, except that the thiol concentration was as indicated and 0.94 unit of enzyme was used.

ance rather than the rate of pyruvate formation, and thus does not involve the effect of pH upon the KDPG aldolase.

Inhibitors—In a reaction mixture containing 6 μmoles of Fe^{++} and 3 μmoles of GSH, inhibitors which combine with metallic ions and with sulfhydryl groups inhibited only when present in excess of the amount required to combine with the Fe^{++} or GSH. Thus 10^{-3} M Versene or cyanide was not inhibitory and 10^{-3} M *p*-chloromercuribenzoate inhibited only 30 per cent. Sodium fluoride, however, inhibited 85 and 100 per cent at 2×10^{-3} M and 5×10^{-2} M, respectively. Further tests were run on dried cells

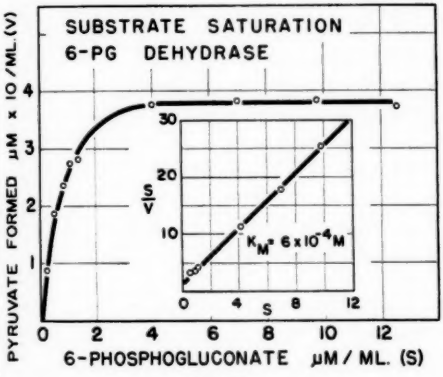


FIG. 5

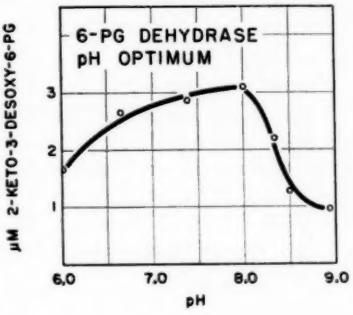


FIG. 6

FIG. 5. The effect of substrate concentration on 6-PG dehydrase activity. The conditions were as in Fig. 1, except that the 6-PG concentration was as indicated.

FIG. 6. The pH optimum for 6-PG dehydrase action. The additions were as in Fig. 1, except that 0.8 unit of dehydrase was added and KDPG aldolase was omitted. Aliquots were removed after 20 minutes incubation and immersed in boiling water for 2 minutes. A suitable dilution was assayed for KDPG.

which convert 6-PG to pyruvate and triose phosphate in the absence of added activators. Under these conditions 10^{-3} M CN^- inhibited 38 per cent, 10^{-3} M *p*-chloromercuribenzoate or Versene 100 per cent, and 10^{-3} M iodoacetate 11 per cent. 5×10^{-3} M sodium arsenite was not inhibitory.

End-Product—The disappearance of 6-PG was not accompanied by pyruvate formation but by the accumulation of an unidentified phosphate ester. This compound already has been postulated to be 2-keto-3-deoxy-6-phosphogluconate (3). The compound migrated slightly more slowly on paper than 6-PG (R_f = 0.43, methanol-ammonia-water, 6:1:3), and was detected by the phosphate ester reagent of Haynes and Isherwood (17), by ultraviolet light absorption after spraying with semicarbazide (18), and by colored spots with *m*-phenylenediamine (19) and *p*-anisidine (20), and by

a positive reducing test with the Somogyi copper reagent (21), followed by the Nelson color reagent (22). These tests indicated that the end-product was a phosphate ester containing a carbonyl group.

With 1-C^{14}-6-PG as the substrate, a C^{14}-labeled product was prepared by incubation with 6-PG dehydrase. The 1-C^{14}-6-PG (143 μM) contained 16,800 c.p.m. After 80 minutes incubation, the reaction was stopped with trichloroacetic acid, and excess of barium acetate was added, the pH was adjusted to pH 3.5, and 4 volumes of ethanol were added. The precipitated barium salt contained 8750 c.p.m. and did not contain pyruvate or 6-PG. The barium was removed, the phosphate ester degraded with ceric sulfate, and the evolved carbon dioxide measured manometrically and absorbed in KOH. The carbon dioxide contained 8650 c.p.m. The complete recovery of the radioactivity as CO_2 after ceric sulfate degradation is evidence that all of the C^{14} was in the carboxyl group of an α-keto acid-phosphate ester whose carboxyl group arose from carbon atom 1 of 6-PG.

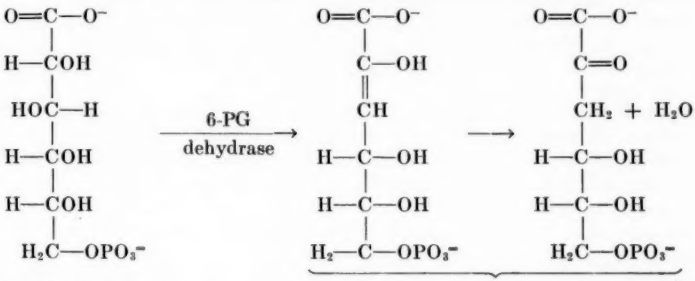
The keto acid-phosphate ester recovered from reaction mixtures as the barium salt was converted to pyruvate and glyceraldehyde-3-phosphate by KDPG aldolase, as was authentic Na_3-2-keto-3-deoxy-6-phosphogluconate (4). Crystalline fructose-1,6-diphosphate aldolase did not cleave the keto acid phosphate ester.

DISCUSSION

The enzymatic attack on 6-PG by the partially purified enzyme resulted in the formation of a compound having the characteristics of 2-keto-3-deoxy-6-phosphogluconate. The conversion of 6-PG as postulated by Entner and Doudoroff (3) would involve the removal of water between carbon atoms 2 and 3, followed by ketoenol tautomerism, as seen in the accompanying diagram. The results obtained are in agreement with the postulated mechanism. Thus until the reaction mechanism is more fully established, the term 6-phosphogluconate dehydrase has been used to designate the enzymatic ability to form KDPG from 6-PG. The requirement for metallic ions and a reducing agent is analogous to requirements demonstrated for aconitase (23).

The discrepancy between the rate of 6-PG utilization and the rate of KDPG formation during the first 10 to 15 minutes of the reaction may be due to the participation of more than one reaction in KDPG formation, with the final reaction governing KDPG formation being slower than the reaction removing 6-PG. The fact that the dehydration is apparently irreversible suggests the existence of a complex mechanism. The data agree with the hypothesis that the tautomeric conversion of enol-KDPG to the keto form is the limiting reaction and is essentially irreversible. This situation may be analogous to the enzymatic oxidation of glucose to glu-

conic acid through δ-gluconolactone. The hydrolysis of the lactone at pH 7 is essentially irreversible. Thus the reduction of gluconic acid by



DPNH has not been observed. However, the reduction of gluconolactone readily occurs.

The presence of large amounts of 6-PG dehydrase and KDPG aldolase (2) relative to 6-PG dehydrogenase in extracts of *P. fluorescens* indicates an important rôle of this alternate pathway in phosphate ester metabolism. This 6-PG-splitting system may afford a means of by-passing, for a large portion of the carbohydrate carbon (pyruvate), the transketolase and transaldolase exchange reactions which are also present in this organism[1] as follows:

$$\text{Glucose} + \tfrac{1}{2}\ \text{oxygen} \rightarrow \text{gluconate} + \text{ATP} \rightarrow \text{6-phosphogluconate}$$
$$\text{6-Phosphogluconate} \rightarrow \text{KDPG} \rightarrow \text{pyruvate} + \text{G-3-P}$$

An active glyceraldehyde-3-phosphate dehydrogenase is present in extracts (3), but beyond this little is known of the mechanism of G-3-P utilization in this organism.

SUMMARY

6-Phosphogluconate dehydrase has been purified 27-fold from vacuum-dried *Pseudomonas fluorescens*. A complete elimination of KDPG aldolase was achieved. An α-keto acid phosphate ester enzymatically identical with authentic 2-keto-3-deoxy-6-phosphogluconate was formed by the action of the dehydrase on 6-PG. The reaction proceeds essentially to completion.

Ferrous ions (4×10^{-3} M) and glutathione (10^{-2} M) were required for maximal activity. Manganous or magnesium ions and cysteine or thioglycolate could partially replace ferrous ions and glutathione respectively.

[1] Krichevsky, M. I., and Wood, W. A., unpublished data.

BIBLIOGRAPHY

1. Wood, W. A., and Schwerdt, R. F., *J. Biol. Chem.*, **201,** 501 (1953).
2. Wood, W. A., and Schwerdt, R. F., *J. Biol. Chem.*, **206,** 625 (1954).
3. Entner, N., and Doudoroff, M., *J. Biol. Chem.*, **196,** 853 (1952).
4. MacGee, J., and Doudoroff, M., *J. Biol. Chem.*, **210,** 617 (1954).
5. Campbell, J. J. R., Norris, F. C., and Norris, M. E., *Canad. J. Res., Sect. C*, **27,** 165 (1949).
6. Wood, W. A., and Horecker, B. L., *Biochem. Preparations*, **3,** 71 (1953).
7. Robison, R., and King, E. J., *Biochem. J.*, **25,** 323 (1931).
8. Horecker, B. L., and Smyrniotis, P. Z., *J. Biol. Chem.*, **196,** 135 (1952).
9. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147,** 415 (1943).
10. Sibley, J. A., and Lehninger, A. L., *J. Biol. Chem.*, **177,** 859 (1949).
11. Horecker, B. L., and Smyrniotis, P. Z., *J. Biol. Chem.*, **193,** 371 (1951).
12. Krebs, H. A., and Johnson, W. A., *Biochem. J.*, **31,** 645 (1937).
13. Kovachevich, R., and Wood, W. A., *J. Biol. Chem.*, **213,** 757 (1955).
14. Van Slyke, D. D., Plazin, J., and Weisiger, J. R., *J. Biol. Chem.*, **191,** 299 (1951).
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193,** 265 (1951).
16. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
17. Haynes, C. S., and Isherwood, F. A., *Nature*, **164,** 1107 (1949).
18. Umbarger, H. E., and Magasanik, B., *J. Am. Chem. Soc.*, **74,** 4253 (1952).
19. Scott, D. B. M., and Cohen, S. S., *J. Biol. Chem.*, **188,** 509 (1951).
20. Katznelson, H., Tanenbaum, S. W., and Tatum, E. L., *J. Biol. Chem.*, **204,** 43 (1953).
21. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100,** 695 (1933).
22. Nelson, N., *J. Biol. Chem.*, **153,** 375 (1944).
23. Dickman, S. R., and Cloutier, A. A., *Arch. Biochem.*, **25,** 229 (1950).

CARBOHYDRATE METABOLISM BY PSEUDOMONAS FLUORESCENS

IV. PURIFICATION AND PROPERTIES OF 2-KETO-3-DEOXY-6-PHOSPHOGLUCONATE ALDOLASE*

By R. KOVACHEVICH and W. A. WOOD

(From the Laboratory of Bacteriology, Department of Dairy Science, University of Illinois, Urbana, Illinois)

(Received for publication, August 19, 1954)

Entner and Doudoroff (1) have reported that extracts of *Pseudomonas saccharophila* degrade 6-phosphogluconate (6-PG) to pyruvate and triose phosphate. A two-step reaction mechanism was postulated as follows:

(1) 6-PG → 2-keto-3-deoxy-6-PG (enol) + H_2O

(2) 2-Keto-3-deoxy-6-PG (keto) → pyruvate + D-glyceraldehyde-3-phosphate

MacGee and Doudoroff (2) have gained evidence in support of the postulated scheme by recovering the product of Reaction 1 as a crystalline trisodium salt and establishing its structure as Na_3-2-keto-3-deoxy-6-phosphogluconate·$2H_2O$.

Extracts of *Pseudomonas fluorescens* catalyze the cleavage of 6-PG to pyruvate and triose phosphate at a rate 10 times faster than the rate of 6-PG dehydrogenase action (3). Thus the reactions leading to pyruvate and triose phosphate formation may constitute a major pathway of 6-PG degradation in this organism. Fractionation of sonic extracts of *P. fluorescens* has yielded a partially purified 6-PG dehydrase (4). This preparation did not produce pyruvate from 6-PG but converted 6-PG to 2-keto-3-deoxy-6-phosphogluconate (KDPG) (Reaction 1). Ferrous ions and glutathione were required for activity.

A second enzyme fraction has been obtained from *P. fluorescens* extracts which is inactive on 6-PG, but which catalyzes an aldolase type cleavage of KDPG to form pyruvate and D-glyceraldehyde-3-phosphate (Reaction 2). A combination of the 6-PG dehydrase with the enzyme fraction to be described converted 6-PG to pyruvate and D-glyceraldehyde-3-phosphate (G-3-P), as reported by Entner and Doudoroff (1).

* This research was supported in part by a grant-in-aid from the National Science Foundation. A preliminary account was presented at the annual meeting of the Society of American Bacteriologists, Pittsburgh, Pennsylvania, May, 1954.

Methods

Large quantities of *P. fluorescens*, A3.12, were grown, harvested, and then dried *in vacuo* as described previously (4, 5).

Materials—2-Keto-3-deoxy-6-phosphogluconate was prepared from 6-PG by the purified 6-PG dehydrase (4) and isolated as the barium salt 88 per cent pure. Such preparations did not contain 6-PG as an impurity. Crystalline Na_3-2-keto-3-deoxy-6-PG was supplied by Dr. M. Doudoroff. Reduced diphosphopyridine nucleotide (DPNH) was prepared by the method of Lehninger (6). Deoxyribose-5-phosphate was prepared from deoxyadenylic acid by the method of Racker (7). 6-PG dehydrase was prepared by the method of Kovachevich and Wood (4). D-Glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-diphosphate aldolase, both recrystallized four times, were prepared by the method of Cori *et al.* (8) and Taylor *et al.* (9), respectively. Crystalline rat liver lactic dehydrogenase (enzyme to protein ratio = 8400 (10)) was supplied by Dr. Carl Vestling.

Determinations—Pyruvate formation by crude fractions was followed colorimetrically by the method of Friedemann and Haugen (11). Under conditions employed, G-3-P and KDPG did not interfere with the method. Pyruvate also was determined enzymatically in the presence of excess lactic dehydrogenase and DPNH by the decrease in optical density at 340 mμ. The procedure was essentially that described for the spectrophotometric enzyme assay.

Enzyme Assay—In the first steps of enzyme purification, the amount of KDPG aldolase was determined colorimetrically in the presence of an excess of 6-PG dehydrase by the rate of pyruvate production from 6-PG. Under these conditions pyruvate was not utilized, and the rate of pyruvate formation was dependent only upon the amount of 2-keto-3-deoxy-6-PG aldolase present. The reaction mixture contained 200 μmoles of tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.65, 3 μmoles of NaGSH, 6 μmoles of $FeSO_4$, 0.7 unit of 6-PG dehydrase, and 2-keto-3-deoxy-6-PG aldolase. After 10 minutes equilibration at 37° and 10 minutes incubation with 6-PG, the reaction was stopped by adding the 2,4-dinitrophenylhydrazine reagent of Friedemann and Haugen (11).

Fractions devoid of DPNH-oxidizing activity were assayed spectrophotometrically. Measurements were made in 0.5 ml. capacity cells having a 1 cm. light path. The reaction mixture contained 50 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 7.65, 167 γ of lactic dehydrogenase, 0.8 μmole of KDPG, 0.098 μmole of DPNH, 0.14 unit or less of KDPG aldolase, and water to 0.5 ml. Since an excess of lactic dehydrogenase was present, the rate of pyruvate formation from KDPG governed the rate of DPNH oxidation (decrease in optical density at 340 mμ). The formation of pyruvate with time and the dependence of the reaction rate

upon enzyme concentration are shown in Fig. 1. Under these conditions the rate of DPNH oxidation was linear over a 3 minute period, and the rate was proportional to KDPG aldolase concentration between 0.01 and 0.14 unit per 0.5 ml. 1 unit of enzyme is defined as the amount of enzyme necessary to form 1 μmole of pyruvate in 10 minutes in the spectrophotometric assay.

Results

Enzyme Fractionation—Preparation of the sonic extract and the first three steps in purification were part of the procedure for preparing 6-PG

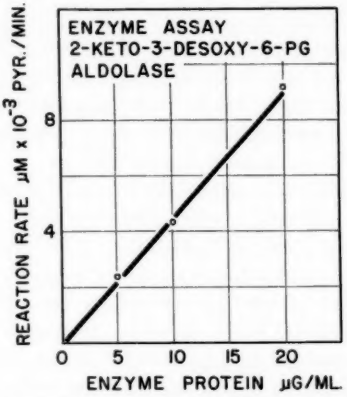


FIG. 1. The rate of pyruvate formation as a function of KDPG aldolase concentration. 0.5 ml. cuvettes (1 = 1 cm.) contained 50 μmoles of Tris buffer, pH 7.65, 0.098 μmole of DPNH, 167 γ of lactic dehydrogenase, 0.8 μmole of KDPG, KDPG aldolase (4.5 units per mg. of protein), and water to 0.5 ml. The rate of pyruvate formation was calculated from the rate of DPNH oxidation over a 3 minute interval.

dehydrase (4). 20 gm. of vacuum-dried *P. fluorescens* were dissolved in 400 ml. of 0.1 M phosphate, pH 7.0, and treated in a 200 watt, 10 kc. sonic oscillator for 5 to 15 minutes. The KDPG aldolase was completely liberated and was found in the soluble protein fraction.

Ammonium Sulfate Precipitation—An ammonium sulfate solution saturated at 1° and neutralized with ammonium hydroxide to pH 7.3 at 2° was used throughout. To each 100 ml. of sonic-treated cell suspension, 49 ml. (0.25 saturation) of saturated ammonium sulfate were added. The precipitate, which contained cellular débris and particles, was separated by centrifugation and discarded. To each 100 ml. of the supernatant solution an additional 84 ml. (0.5 saturation) of saturated ammonium sulfate were added. The precipitate was recovered by centrifugation and dissolved in 100 ml. of 0.1 M phosphate buffer, pH 7.0.

Protamine Precipitation—20 to 25 ml. of protamine sulfate, pH 5.0, were added to each 100 ml. of enzyme solution. The precipitate which formed immediately was removed by centrifugation. The ratio of absorption at 280 and 260 mμ was approximately 0.9.

Ammonium Sulfate Precipitation—To each 100 ml. of supernatant solution from the protamine treatment, 113 ml. (0.40 saturation) of saturated ammonium sulfate were added. The precipitate was sedimented by centrifugation and dissolved in 37 ml. of 0.1 M phosphate buffer, pH 7. This fraction contained all of the 6-PG dehydrase activity and appreciable amounts of KDPG aldolase. To each 100 ml. of the supernatant solution 27 ml. of ammonium sulfate were added (0.5 saturation). After centrifugation the precipitate was redissolved in 51 ml. of 10^{-2} M Tris buffer, pH 7.65. This fraction was devoid of 6-PG dehydrase activity and was stable in the absence of phosphate. The fraction was dialyzed 20 hours against 10^{-2} M Tris buffer, pH 7.0, and stored for 2 weeks at $-15°$ before proceeding to the next step. No activity was lost during storage.

Calcium Phosphate Gel Adsorption and Elution—Portions of gel suspension (37 mg. per ml., dry weight) were added to the 0.4 to 0.5 ammonium sulfate fraction, and the solution was stirred for 10 minutes in an ice bath. The gel was removed by centrifugation, and the supernatant fluid was assayed for activity. Aliquots of gel were added until the activity began to disappear in the supernatant fluid. Six 3 ml. portions of gel were added in this manner and discarded. An additional 5.1 ml. of gel were added to the supernatant solution, stirred for 10 minutes, and collected by centrifugation. This portion, which removed 80 per cent of the activity from the solution, was washed with 17 ml. of distilled water, centrifuged, and the washing discarded. The activity was eluted by stirring the gel for 30 minutes with 17 ml. of 0.01 M phosphate buffer, pH 7.6. The eluate contained KDPG aldolase purified 25- to 26-fold over the crude extract and was free of 6-PG dehydrase (Table I).

Properties of 2-Keto-3-deoxy-6-phosphogluconate Aldolase—The action of KDPG aldolase in two buffers is shown in Fig. 2. In Tris buffer, pH 7.6, the disappearance of KDPG was accompanied by the formation of an equivalent amount of pyruvate. The reaction proceeded until virtually all of the KDPG was utilized. However, only a small amount of G-3-P could be detected during the reaction, suggesting that G-3-P was undergoing further reactions. Additional tests showed that analyses for G-3-P in Tris buffer were satisfactory if carried out immediately. However, after a short incubation time with Tris buffer, the values were either low or G-3-P could not be detected at all. The reaction between amino compounds such as tris(hydroxymethyl)aminomethane and G-3-P, reported by Segal and Boyer (12), probably accounts for the inability to demonstrate G-3-P formation in amounts equivalent to the pyruvate formed.

TABLE I

Purification of 2-Keto-3-deoxy-6-phosphogluconate Aldolase from Vacuum-Dried P. fluorescens

Step No.		KDPG aldolase	Specific activity, units per mg. of protein	KDPG aldolase / 6-PG dehydrase
		units		
1	Sonic extract	29,600	4.5	2.8
2	0.25–0.50 saturated $(NH_4)_2SO_4$ ppt.	26,000	7.6	3.7
3	Protamine supernatant	17,600	5.4	2.2
4	0.4–0.5 saturated $(NH_4)_2SO_4$ ppt.	9,400	20.0	∞
5	Calcium phosphate gel eluate	1,110	112.0	∞

With sodium β-glycerol phosphate hydrochloric acid buffer, pH 7.0, the disappearance of KDPG was initially accompanied by the formation of an equivalent amount of both pyruvate and G-3-P. Even under these conditions a disappearance of G-3-P was observed. It can be seen, however, that product formation and KDPG disappearance ceased when about 64 per cent of the KDPG had been utilized. These data suggest that an enzymatic degradation of G-3-P occurs. Thus further purification is necessary before the equilibrium point of the reaction can be established.

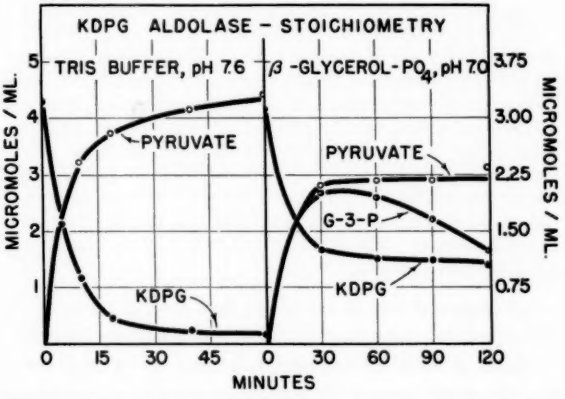


FIG. 2. Stoichiometry of KDPG aldolase action. In the left-hand section the 1 ml. reaction mixture contained 200 μmoles of Tris buffer, pH 7.65, 4.3 μmoles of KDPG, and 2 units of KDPG aldolase. Aliquots of the reaction mixture were removed at the times indicated, the reaction was stopped by boiling for 2 minutes, and aliquots were removed for pyruvate and KDPG determinations. In the right-hand section the conditions were the same, except that the reaction mixture contained 100 μmoles of β-glycerol phosphate hydrochloride buffer, pH 7, and 3.15 μmoles of KDPG. The reaction was stopped by adding trichloroacetic acid.

The nature of the reaction products was demonstrated from the enzymatic reactions shown in Fig. 3. In a reaction mixture containing lactic dehydrogenase, DPNH, DPN, arsenate, and glutathione, the addition of 0.17 μmole of KDPG did not cause a decrease in optical density (left-hand section). However, upon addition of KDPG aldolase a decrease in

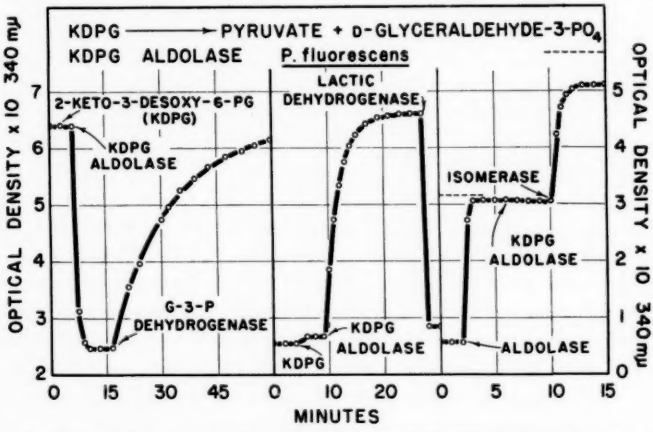


FIG. 3. The identification of the end-products of KDPG aldolase action. In the left-hand section, the 3 ml. reaction mixture contained 500 γ of lactic dehydrogenase, 300 μmoles of Tris buffer, pH 7.65, 0.14 μmole of DPNH, 0.14 μmole of DPN, 6 μmoles of sodium arsenate, 12 μmoles of NaGSH, 0.17 μmole of KDPG, 3 units of KDPG aldolase, and 34 γ of crystalline G-3-P dehydrogenase. The KDPG, KDPG aldolase, and G-3-P dehydrogenase were added at the times indicated. The conditions were the same for the center section, except that DPNH was not added, and KDPG, KDPG aldolase, and lactic dehydrogenase were added at the times indicated. In the right-hand section the test conditions were those of the center section, except that the 3 ml. reaction mixture contained 3.4 mg. of G-3-P dehydrogenase, 500 γ of crystalline aldolase, 0.127 μmole of fructose-1,6-diphosphate, and 0.03 ml. of partially purified triose phosphate isomerase. The aldolase, KDPG aldolase, and triose phosphate isomerase were added as indicated.

optical density occurred which corresponded to 0.172 μmole of pyruvate formed. Following this, the addition of glyceraldehyde-3-phosphate dehydrogenase caused an increase in optical density which was approximately equal to the decrease caused by pyruvate formation (action of KDPG aldolase and lactic dehydrogenase on KDPG). The action of G-3-P dehydrogenase was slow, presumably due to the interference by Tris buffer. When the G-3-P dehydrogenase was added first, followed by KDPG and KDPG aldolase, a rapid formation of DPNH occurred (center section). The amount was equivalent to the amount of KDPG added. The addition of lactic dehydrogenase then caused a rapid oxidation of DPNH

and a decrease in optical density to approximately the original value. These tests revealed that equivalent amounts of pyruvate and a triose phosphate were produced. From the data in the right-hand section it was established that the KDPG aldolase preparation was devoid of triose phosphate isomerase activity. In the presence of 4 times recrystallized fructose-1,6-diphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase, the addition of 0.127 μmole of fructose-1,6-diphosphate (F-1,6-P) caused the generation of an equimolar amount of DPNH. This was one-half the amount expected if isomerase was present and the oxidation of dihydroxyacetone phosphate had occurred. The addition of KDPG aldolase after DPNH formation had ceased did not cause further DPNH formation. The inability of the KDPG aldolase preparation to catalyze the conversion of dihydroxyacetone phosphate to G-3-P indicates the absence of triose phosphate isomerase in the preparation. The addition of a partially purified triose phosphate isomerase (13) prepared from rabbit muscle to the same cuvette did cause the formation of an additional DPNH in approximately the amount expected for the oxidation of the second triose phosphate. The reduction of DPN by the combined action of glyceraldehyde-3-phosphate dehydrogenase and the triose phosphate isomerase-free KDPG aldolase, in an amount equal to the pyruvate formed, establishes the identity of the triose phosphate formed from KDPG as D-glyceraldehyde-3-phosphate.

Substrate—The effect of substrate concentration upon the reaction rate is shown in Fig. 4. Maximal velocity was obtained with 1.6 μmoles of KDPG per ml. The concentration necessary for one-half maximal activity was calculated from the plots according to Lineweaver and Burk (14) to be about 0.1 μmole per ml. ($K_m = 1.1 \times 10^{-4}$ M). Fructose-1,6-diphosphate and deoxyribose-5-phosphate were not split by KDPG aldolase, as evidenced by the absence of DPN reduction in reaction mixtures containing these phosphate esters, KDPG aldolase, G-3-P dehydrogenase, DPN, arsenate, and glutathione.

pH Optimum—The dependence of the reaction rate upon pH is shown in Fig. 5. A broad maximum between pH 7.5 and 8.5 was found. In addition, over 50 per cent of the maximal rate was retained at pH 6.3 and 9.5. Essentially similar results were obtained with different buffers.

Coenzyme—No coenzyme requirement has been observed. The enzyme retains full activity after either prolonged dialyses, precipitation with ammonium sulfate, or treatment with Dowex 1 formate or activated carbon. No increase in rate was obtained by adding metallic ions or glutathione. Agents forming metal complexes such as Versene and compounds such as *p*-chloromercuribenzoate, iodoacetate, and arsenite which inhibit by reacting with thiol groups were not inhibitory.

Determination of 2-Keto-3-deoxy-6-phosphogluconate—The coupling of KDPG aldolase with lactic dehydrogenase affords a rapid and accurate

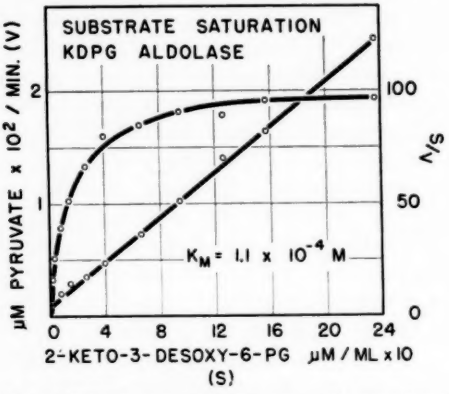


FIG. 4. The effect of KDPG concentration upon the reaction rate. The 0.5 ml. reaction mixture contained 50 μmoles of Tris buffer, pH 7.65, 0.098 μmole of DPNH, 0.13 unit of KDPG aldolase, 167 γ of lactic dehydrogenase, and KDPG as indicated. A plot of the reaction rate (V) *versus* the ratio of the substrate concentrate to reaction rate (S/V) is given by the straight line.

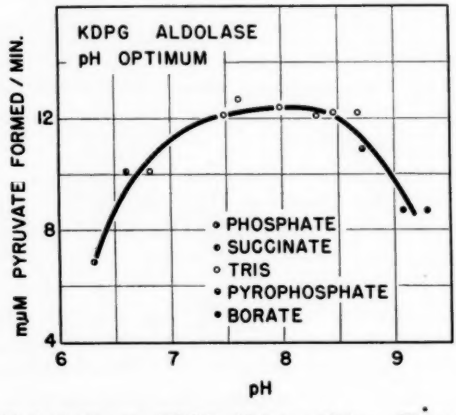


FIG. 5. The effect of pH upon KDPG aldolase activity. The conditions were the same as in Fig. 4, except that 0.4 μmole of KDPG was used. The type of buffer and the pH of each test were as indicated.

optical method for KDPG determination. In the presence of both enzymes and DPNH, the formation of pyruvate is accompanied by a decrease in optical density at 340 mμ. Since pyruvate is rapidly removed, the

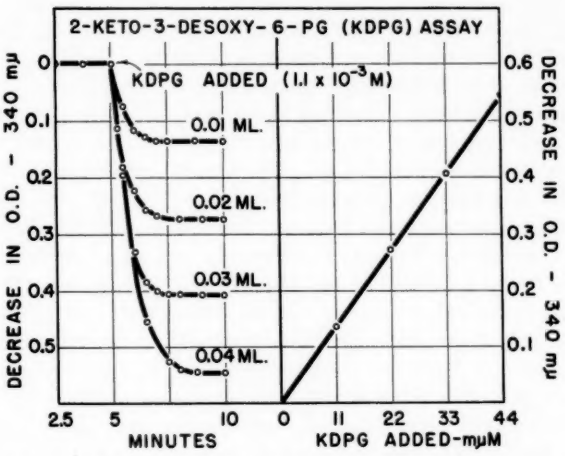


FIG. 6. An optical assay for 2-keto-3-deoxy-6-phosphogluconate. The 0.5 ml. capacity cuvette (1 = 1 cm.) contained 50 μmoles of Tris buffer, pH 7.65, 0.098 μmole of DPNH, 1 unit of KDPG aldolase, 167 γ of lactic dehydrogenase, and KDPG as indicated.

equilibrium is shifted and KDPG is completely converted to pyruvate and G-3-P. Fig. 6 shows the optical density changes with 1.1 to 4.4 × 10^{-3} mole of KDPG in a 0.5 ml. cuvette. This assay can be performed only with preparations which do not contain DPNH oxidase activity. Assays of

TABLE II

Distribution of 6-PG Dehydrase and KDPG Aldolase in Bacteria

The conditions were as described for the colorimetric enzyme assay. Sonic extracts were tested with 6-PG, 5 μmoles per ml., and dried cells with KDPG, 2 μmoles per ml.

Organism	Pyruvate per mg. protein per hr.	
	6-PG	KDPG
	μmoles	*μmoles*
P. fluorescens, A-3.12	3.2	6.6
" *aeruginosa* ATCC 9027	2.5	6.1
" *fragi* UIDS 215	2.5	0.3
" *putrefaciens* UI 206	0	0.1
Acetobacter suboxydans NRRL B-72	1.9	0.9
E. coli ATCC 9739	1.0	6.6
Proteus vulgaris WDB 47	0	0.2

crystalline Na_3-2-keto-3-deoxy-6-phosphogluconate·$2H_2O$ by this method demonstrated a 100 per cent conversion to pyruvate.

Distribution of 6-PG-Splitting System and KDPG Aldolase in Bacteria—Table II shows the 6-PG dehydrase and KDPG aldolase content of dried cells and sonic extracts of several gram-negative glucose-grown bacteria. The activity was measured by the rate of pyruvate formation with 6-PG and KDPG as substrates and was determined only with organisms which did not utilize pyruvate under the assay conditions. High 6-PG dehydrase and KDPG aldolase activity (1 to 6.6 μmoles per mg. of protein per hour) was shown by both dried cells and extracts of *P. fluorescens*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Sonic extracts of *Pseudomonas fragi* rapidly formed pyruvate from 6-PG, whereas dried cells were low in KDPG aldolase activity. *Acetobacter melanogenum*[1] and *Azotobacter vinelandii*[2] also possess active KDPG aldolase and 6-PG dehydrase. Pyruvate formation from 6-PG was not observed with *Pseudomonas putrefaciens* and *Proteus vulgaris*. The KDPG aldolase activity was relatively lower (0.1 to 0.3 μmole per mg. dry weight per hour), but could easily be detected. KDPG aldolase was not detected in *Leuconostoc mesenteroides* 8081; *Corynebacterium creatinovorans* 7562; *Lactobacillus arabinosus* 8014; *Microbacterium lacticum* 8181;[3] and brewers' yeast.

DISCUSSION

A combination of partially purified 6-PG dehydrase and KDPG aldolase catalyzes the conversion of 6-PG to pyruvate and G-3-P. The separation of the reaction forming KDPG (4) and the isolation, characterization (2), and quantitative conversion of KDPG to pyruvate and G-3-P constitute evidence in favor of the reaction mechanism postulated by Entner and Doudoroff (1). However, data are not available as to whether a keto-enol tautomeric reaction of KDPG is an obligatory part of the reaction sequence.

The cleavage of 2-keto-3-deoxy-6-phosphogluconate to pyruvate and D-glyceraldehyde-3-phosphate resembles the conversion of deoxyribose-5-phosphate to D-glyceraldehyde-3-phosphate and acetaldehyde by the deoxyribose phosphate (DR) aldolase described by Racker (7). The enzyme which splits KDPG is distinct from DR aldolase and F-1,6-P aldolase, however, since it does not cleave deoxyribose-5-phosphate or F-1,6-P. For this reason the enzyme has been termed 2-keto-3-deoxy-6-phosphogluconate aldolase. The equilibria of the cleavage reactions catalyzed by deoxyribose phosphate aldolase and fructose diphosphate aldolase ap-

[1] Katznelson, H., and Wood, W. A., unpublished data.
[2] Mortenson, L. E., and Wilson, P. W., private communication.
[3] VanDemark, P. J., and Wood, W. A., unpublished data.

parently differ from that of KDPG aldolase. In the former cases the equilibria favor the condensation product, whereas with KDPG aldolase the equilibrium is toward the cleavage products.

Assay of dried cells of several species for KDPG aldolase revealed the existence of this pathway of 6-PG utilization in several gram-negative organisms. It is noteworthy that gram-positive organisms such as bacilli, streptococci, lactobacilli, and microbacterium do not contain this system. However, it is likely many other organisms may also contain this pathway of 6-PG degradation when grown and tested under the proper conditions.

SUMMARY

2-Keto-3-deoxy-6-phosphogluconate aldolase has been purified 25- to 26-fold from extracts of vacuum-dried *Pseudomonas fluorescens*. The preparation was completely devoid of 6-PG dehydrase, fructose diphosphate aldolase, deoxyribose phosphate aldolase, and triose phosphate isomerase. KDPG aldolase catalyzed the conversion of 2-keto-3-deoxy-6-phosphogluconate to equimolar amounts of pyruvate and D-glyceraldehyde-3-phosphate. The equilibrium appears to be approximately 64 per cent in the direction of pyruvate and G-3-P formation. No cofactor requirement could be demonstrated, and the enzyme was relatively inert toward sulfhydryl inhibitors and agents forming metal complexes. The enzyme requires approximately 0.1 μmole of KDPG per ml. for half maximal velocity. A broad pH optimum between pH 7.0 and 8.5 was found. An optical method for KDPG determination with KDPG aldolase is described. A limited survey of bacteria revealed the presence of KDPG aldolase in several gram-negative bacteria, but the enzyme could not be detected in gram-positive organisms.

BIBLIOGRAPHY

1. Entner, N., and Doudoroff, M., *J. Biol. Chem.*, **196,** 853 (1952).
2. MacGee, J., and Doudoroff, M., *J. Biol. Chem.*, **210,** 617 (1954).
3. Wood, W. A., and Schwerdt, R. F., *J. Biol. Chem.*, **206,** 625 (1954).
4. Kovachevich, R., and Wood, W. A., *J. Biol. Chem.*, **213,** 745 (1955).
5. Wood, W. A., and Schwerdt, R. F., *J. Biol. Chem.*, **201,** 501 (1953).
6. Lehninger, A. L., *Biochem. Preparations*, **2,** 92 (1952).
7. Racker, E., *J. Biol. Chem.*, **196,** 347 (1952).
8. Cori, G. T., Slein, M. W., and Cori, C. F., *J. Biol. Chem.*, **173,** 605 (1948).
9. Taylor, J. F., Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **173,** 591 (1948).
10. Gibson, D. M., Davisson, E. O., Bachhawat, B. K., Ray, B. R., and Vestling, C. S., *J. Biol. Chem.*, **203,** 397 (1953).
11. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147,** 415 (1943)
12. Segal, H. L., and Boyer, P. D., *J. Biol. Chem.*, **204,** 265 (1953).
13. Meyerhof, O., and Beck, L. V., *J. Biol. Chem.*, **156,** 109 (1944).
14. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).

THE METABOLIC BEHAVIOR OF REDUCED GLUTATHIONE IN HUMAN AND AVIAN ERYTHROCYTES*

By E. DIMANT,† EDITH LANDSBERG, AND IRVING M. LONDON

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York, New York)

(Received for publication, August 26, 1954)

In studies in this laboratory on the process of aging in mammalian and avian erythrocytes, the metabolic behavior of various constituents of these erythrocytes is being investigated (2, 3). This report is concerned with the pattern of turnover of reduced glutathione, GSH, in human erythrocytes *in vivo* and with the incorporation of glycine into the GSH of human and duck erythrocytes *in vitro*.

Investigation of the pattern of turnover of the GSH of human erythrocytes was designed to determine whether this GSH is in the dynamic state or whether, once incorporated into the newly formed erythrocyte, it is stable metabolically and remains with the erythrocyte throughout the life of the erythrocyte, as does hemoglobin (2, 4–6). This problem is related to the question of the origin of the GSH in mammalian blood, a question which continues to be the subject of much speculation. Some workers have suggested that the GSH is synthesized in the liver and subsequently incorporated into the circulating erythrocyte (7). GSH in human blood, however, has been shown to exist within erythrocytes (8) and leucocytes (9); there is no measurable GSH in human plasma nor is there as yet evidence to indicate the permeability of the erythrocyte membrane to the GSH of the surrounding medium. Others have suggested that the GSH is synthesized in the newly formed erythrocyte (10). It has also been suggested that some turnover of GSH occurs in the mature erythrocyte (11). It is possible that GSH may be formed in the immature erythrocyte and that subsequently it may undergo turnover in the circulating erythrocyte as a result of exchange of one or more of the constituents of GSH. Finally, the possibility must be considered that GSH is continually renewed in the circulating erythrocytes as a result of synthesis of GSH *de novo* within the cell.

* A report of this work was submitted to the American Society for Clinical Investigation at Atlantic City, May, 1954 (1). The work was supported by a contract between the Office of Naval Research and Columbia University, NR 26616, and by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

† Eli Lilly Research Fellow, 1952–53.

XUM

The incorporation of glycine into the GSH of human and duck erythrocytes *in vitro* was studied with $C^{14}H_2NH_2COOH$ and into the GSH of human erythrocytes *in vivo* with $CH_2N^{15}H_2COOH$. In the experiment in man, the survival of the erythrocytes was evaluated by determining the isotope concentrations in the hemin isolated from the erythrocytes at intervals during the experiment (2, 5).

Materials and Methods

Blood—Avian blood was obtained by exsanguination of white Pekin ducks, and human blood samples were obtained by venipuncture. Heparin was employed as an anticoagulant. When erythrocytes or hemolysates were incubated *in vitro*, the plasma was first removed by centrifugation. Hemolysates were prepared by the addition to the erythrocytes of deionized water equal in volume to the plasma removed, and the hemolysates were then made isotonic (0.31 M) by the addition of sucrose. In some experiments hemolysates were centrifuged in a refrigerated Servall centrifuge at 23,000 × *g* at 4° for 15 minutes, and the posthemolytic residue and the supernatant solution so prepared were studied separately.

The glycine-2-C^{14} was obtained from the Nuclear Instrument and Chemical Corporation, Chicago, and was diluted with non-radioactive glycine to yield a specific radioactivity of 0.1 mc. per mmole. N^{15}-labeled glycine was synthesized from potassium phthalimide (12).

Incubation—Samples of whole blood, suspensions of erythrocytes in isotonic sucrose, hemolysates, posthemolytic residues, and soluble supernatant portions of hemolysates were incubated with glycine-2-C^{14} ($C^{14}H_2 \cdot NH_2$—COOH, 0.1 mc. per mmole) at room temperature for periods up to 24 hours with constant, gentle shaking. Penicillin and streptomycin were added to inhibit bacterial growth.

Isolation and Counting—Reduced glutathione was isolated as the cuprous mercaptide according to the procedure of Waelsch and Rittenberg (13). The samples of cuprous mercaptide were weighed, suspended in water, and decomposed with H_2S. After removal of the copper sulfide by centrifugation, residual H_2S was removed from the supernatant solution with a stream of nitrogen. A measured volume of the supernatant solution was then put on stainless steel planchets on which it was evaporated to dryness in a vacuum desiccator at room temperature. The GSH content of the solution was measured according to the procedure of Lazarow, Patterson, and Cooperstein.[1] In this procedure, GSH reacts maximally with alloxan at pH 7.5 to form a compound with a maximal absorption at 305 mμ. The reaction is allowed to proceed for 6 minutes, at which time the mixture is made strongly alkaline with 1.0 N NaOH. At alkaline pH, the

[1] A. Lazarow, J. W. Patterson, and S. J. Cooperstein, personal communication.

product of the reaction is stable and its concentration is determined by measuring the absorbance at 305 mμ in the Beckman DU spectrophotometer.

Electrophoresis on some of the supernatant solutions was carried out on Whatman No. 3 filter paper in an acetate buffer of pH 4.5 and ionic strength 0.005 for 2 hours at 800 volts with a current of about 4 ma. The papers were dried at 105° for 30 minutes and then stained with ninhydrin.[2]

Measurements of C^{14} activity, both of the glycine-2-C^{14} and of the GSH, were made at infinite thinness with a thin window Geiger-Müller tube. The glycine-2-C^{14} was diluted with non-isotopic glycine prior to measurement of its radioactivity.

Experiment in Man—3 gm. of N^{15}-labeled glycine (34 atom per cent excess N^{15}), followed by eight portions of 1.5 gm. of N^{15}-labeled glycine, were administered orally to a normal man at intervals of half an hour. 50 ml. samples of venous blood were drawn at the intervals indicated in Fig. 1, after the first ingestion of labeled glycine. The first sample was drawn at 6 hours. Plasma and the buffy coat of leucocytes were removed after centrifugation. The erythrocytes were hemolyzed with approximately 25 ml. of deionized water and the proteins were precipitated with 50 ml. of 10 per cent trichloroacetic acid. The cuprous mercaptide was isolated, washed three times with 0.5 N H_2SO_4, and four times with deionized water, and the N^{15} concentration was determined by the usual procedure (14).

Hemin was isolated directly from erythrocytes (15) or from an HCl-acetone (1.2 ml. of concentrated HCl-100 ml. of acetone) extract of the trichloroacetic acid precipitate formed in the isolation of GSH. All samples of hemin were recrystallized (16) prior to analysis of isotope concentration.

RESULTS AND DISCUSSION

Table I, Samples 1 to 7, and Table II present evidence for the existence of a mechanism by which the methylene carbon of glycine can be incorporated into the GSH of duck erythrocytes and of human erythrocytes *in vitro*. This mechanism exists not only in intact erythrocytes, but also in lyzed erythrocytes and in the posthemolytic residue of these cells. Under the conditions of these experiments, some incorporation of the methylene carbon of glycine into GSH was observed in the soluble supernatant portion of the hemolysate of human erythrocytes, but not in that of duck erythrocytes.

Table I, Samples 7 to 9, presents evidence that the radioactivity in the

[2] We are indebted to Dr. Heinrich Waelsch for advice in performing the electrophoretic analyses.

TABLE I

Incorporation of Methylene Carbon of Glycine into Glutathione of Duck Erythrocytes in Vitro

Sample No.	System	C^{14} radioactivity of glycine added, c.p.m. per ml. of system	R. I. C.*
1	Whole blood (90 ml.)†	2.25×10^4	0.034
2	40 ml. erythrocytes in isotonic sucrose (100 ml.)	2.02×10^4	0.033
3	40 " " lyzed with 60 ml. H_2O, isotonicity restored with sucrose (100 ml.)	2.02×10^4	0.037
4	Supernatant of hemolysate of (3) (60 ml.)	3.38×10^4	0
5	Hemolysate as in (3) (90 ml.)	2.25×10^4	0.033
6	Residue of (5) + 10 ml. 0.31 M sucrose (45 ml.)	4.50×10^4	0.048
7	29 ml. erythrocytes lyzed with 43 ml. H_2O, isotonicity restored with sucrose (72 ml.)	9.40×10^3	0.025
8	As in (7)	9.40×10^3	0.00009
9	" " (7)	9.40×10^3	0

Samples 1 to 4 were incubated at room temperature for 17.5 hours; Samples 5, 6, and 7 were incubated at room temperature for 24 hours. Sample 8 was placed in the deep freeze (−15°) immediately after addition of labeled glycine. Sample 9 was deproteinized immediately after addition of labeled glycine.

* R. I. C. is the relative isotope concentration, *i.e.* the ratio of the radioactivity, expressed in counts per minute per millimole, of the GSH isolated to the radioactivity, expressed in counts per minute per mmole, of the glycine-2-C^{14} added.

† The figures in parentheses indicate the total volume of each sample.

TABLE II

Incorporation of Methylene Carbon of Glycine into Glutathione of Human Erythrocytes in Vitro

Sample No.	System*	C^{14} radioactivity of glycine added, c.p.m. per ml. of system	R. I. C.*
A-1	27.5 ml. erythrocytes lyzed with 42.5 ml. H_2O, isotonicity restored with sucrose (70 ml.)	1.93×10^4	0.055
B-2	27 ml. residue of 120 ml. hemolysate + 66 ml. 0.31 M sucrose (93 ml.)	2.18×10^4	0.139
B-3	Supernatant of 120 ml. hemolysate (93 ml.)	2.18×10^4	0.012
C-4	22 ml. erythrocytes in isotonic sucrose (55 ml.)	2.45×10^4	0.191
D-5	27.5 ml. residue of 120 ml. hemolysate + 72.5 ml. 0.31 M sucrose (100 ml.)	2.02×10^4	0.150
D-6	Supernatant of 120 ml. hemolysate (95 ml.)	2.13×10^4	0.019

Samples A-1, B-2, and B-3 were incubated for 16 hours and Samples C-4, D-5, and D-6 for 18 hours, all at room temperature.

* See foot-notes to Table I.

GSH of duck erythrocytes which have been incubated with glycine-2-C^{14} is not an artifact due to adsorption or coprecipitation of isotopic material other than GSH. GSH isolated from duck erythrocytes immediately after the addition of labeled glycine or from duck erythrocytes which were frozen immediately after addition of labeled glycine was not radioactive. Similar samples of duck erythrocytes incubated at room temperature with labeled glycine showed good incorporation of the methylene carbon of glycine into GSH. Analysis by paper electrophoresis of some of the super-

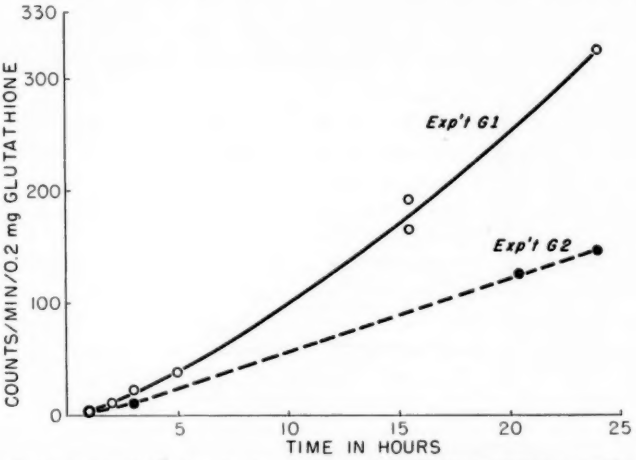


FIG. 1. Incorporation of methylene carbon of $C^{14}H_2NH_2COOH$ into GSH of duck erythrocytes. In Experiment G1, 75 ml. samples of whole blood containing 12 mg. of $C^{14}H_2NH_2COOH$, 0.1 mc. per mmole, and in Experiment G2, 100 ml. samples of whole blood containing 12 mg. of $C^{14}H_2NH_2COOH$, 0.1 mc. per mmole, were incubated for varying periods of time.

natant solutions of GSH which were used for assay and counting did not reveal the presence of any constituents other than GSH and oxidized glutathione.

Fig. 1 shows the progressive increase, with time, of the specific radioactivity of GSH of hemolyzed preparations of duck erythrocytes which were incubated with glycine-2-C^{14}. These findings indicate that the mechanism responsible for the incorporation of the methylene carbon of glycine into GSH in this system is stable at room temperature for at least 24 hours.

The results of the experiment in a man are presented in Fig. 2. The N^{15} concentrations in the cuprous mercaptide of glutathione isolated serially from the erythrocytes rise rapidly to a maximal concentration in about 24 hours and then decline almost exponentially. The N^{15} concentrations

in the hemin reveal that the erythrocytes have an average life span of over 4 months. (A more precise estimate of the erythrocyte life span has not been attempted because the experimental data are too few for greater precision.) These findings indicate that the GSH of circulating human erythrocytes is in the dynamic state, in contrast to the hemoglobin of these cells which is not in the dynamic state. The nitrogen of the glutathione of the erythrocytes which is derived from the nitrogen of the administered $CH_2N^{15}H_2COOH$ has a half life time of approximately 4 days. It should be noted, in light of the findings of Waelsch and Rittenberg (13), that the N^{15} of the isolated GSH is, most likely, not restricted to the nitrogen of glycine but is also found in the glutamyl and cysteinyl moieties. The

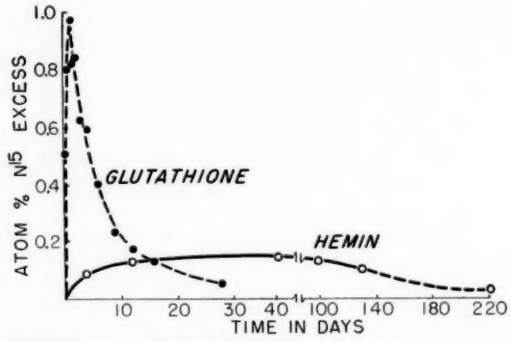


FIG. 2. N^{15} concentrations in GSH and hemin of erythrocytes after start of feeding of $CH_2N^{15}H_2COOH$ over a 4 hour period.

value for the half life time is, accordingly, only an approximation of the rate of turnover of glutathione in the circulating erythrocyte.

The demonstrations that the GSH of circulating human erythrocytes is in the dynamic state and that these cells *in vitro* can incorporate the methylene carbon of glycine into their GSH are pertinent to the question of the origin of the GSH of these erythrocytes. It is assumed that the incorporation of the methylene carbon of glycine is indicative of the incorporation of glycine into GSH. The incorporation of glycine into GSH may represent either exchange of glycine in preformed GSH or *de novo* synthesis of the cysteinylglycine peptide bond or both. It remains to be determined whether both processes are present in human erythrocytes and, if so, whether they are functions of a single enzyme, as is true for the enzyme from yeast, but not for that from pigeon liver (17). These considerations and the absence of GSH in the plasma suggest the possibility that the GSH in human erythrocytes may be formed *in situ* and that one need not postu-

late a hepatic origin for it. Indeed, the additional possibility is raised that at least a portion of the GSH of other tissues may be formed *in situ*.

The finding that the GSH of human erythrocytes is in the dynamic state should be considered in interpreting changes in the concentration of GSH in erythrocytes such as have been reported in sickle-cell anemia (18), diabetes mellitus (19, 20), and untreated pernicious anemia (21, 22). The amount of GSH in erythrocytes is dependent not only on the rates of formation and destruction of GSH, but also on the capacity of the erythrocytes for maintaining glutathione in the reduced form. The reduction of oxidized glutathione is catalyzed by a specific enzyme which has been found in human erythrocytes (23) as well as in other tissues.

Of particular interest in considering the possible rôle of GSH in maintaining the integrity of the erythrocyte are studies which tend to relate GSH and sulfhydryl groups to hemolysis. Working with horse erythrocytes, Fegler (24) found that exposure of the cells to oxygen or to iodine resulted in an increased rate of hemolysis and that the rate of hemolysis increased rapidly after the GSH content of the erythrocytes fell below 40 per cent of the initial value. It should be emphasized, however, that this inverse relationship of GSH content to hemolysis is not necessarily a causal relationship. Benesch and Benesch have shown that organic mercurial compounds can be hemolytic *in vitro* (25). The concentration of phenylmercuric hydroxide required to produce lysis of sheep cells corresponds to the total number of available sulfhydryl groups in the erythrocytes, of which the sulfhydryl groups of hemoglobin constitute about 90 per cent, and those of GSH about 10 per cent. The hemolytic effect can be abolished by prior addition to the system of an amount of GSH equivalent to the mercurial used. Labarre has reported that the hemolytic effect of cobra venom injected into rabbits can be abolished by simultaneous injection of the venom mixed with GSH or by prior injection of GSH (26).

The finding that GSH in human erythrocytes is in the dynamic state is pertinent to the availability of GSH and of other thiols for participation in the maintenance of the structural integrity of the erythrocyte. If GSH is required for the integrity of the erythrocyte, the viability of this cell will depend at least in part on a continued capacity of the cell to replace GSH and to maintain it in a reduced state.

We are deeply indebted to Mrs. Halina Morell for expert help in this work.

SUMMARY

1. Reduced glutathione in the circulating erythrocytes of a normal man is found to be in the dynamic state. After the administration of N^{15}-

labeled glycine, the nitrogen of the reduced glutathione of the erythrocytes which is derived from the administered glycine is shown to have a half life time of approximately 4 days.

2. Incubation of human erythrocytes or of duck erythrocytes with glycine-2-C^{14} *in vitro* results in the incorporation of the labeled carbon into the reduced glutathione. This incorporation occurs not only in intact erythrocytes, but also in lyzed erythrocytes, and, for the most part, in the posthemolytic residues of these cells.

3. The implications of these findings for the origin of the glutathione of the erythrocyte are indicated. The possible relevance of these findings to the maintenance of the structural integrity of the erythrocyte is considered.

BIBLIOGRAPHY

1. Dimant, E., Landsberg, E., and London, I. M., *J. Clin. Invest.*, **33**, 928 (1954).
2. London, I. M., Shemin, D., West, R., and Rittenberg, D., *J. Biol. Chem.*, **179**, 463 (1949).
3. London, I. M., and Schwarz, H., *J. Clin. Invest.*, **32**, 1248 (1953).
4. Jope, E. M., *Brit. J. Ind. Med.*, **3**, 136 (1946).
5. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **166**, 627 (1946).
6. Grinstein, M., Kamen, M. D., and Moore, C. V., *J. Biol. Chem.*, **179**, 359 (1949).
7. Anderson, E. I., and Mosher, W. A., *J. Biol. Chem.*, **188**, 717 (1951).
8. Woodward, G. E., and Fry, E. G., *J. Biol. Chem.*, **97**, 465 (1932).
9. McKinney, G. R., *Arch. Biochem. and Biophys.*, **46**, 246 (1953).
10. Lemberg, R., and Legge, J. W., Hematin compounds and bile pigments, New York, 517 (1949).
11. Mortensen, R. A., *J. Biol. Chem.*, **204**, 239 (1953).
12. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939).
13. Waelsch, H., and Rittenberg, D., *J. Biol. Chem.*, **139**, 761 (1941).
14. Sprinson, D. B., and Rittenberg, D., *J. Biol. Chem.*, **180**, 707 (1949).
15. Fischer, H., *Org. Syntheses*, **21**, 53 (1941).
16. Kuster, W., in Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, Berlin and Vienna, Abt. I, Teil 8, 208 (1922).
17. Snoke, J. E., and Bloch, K., in Colowick, S. P., Glutathione, New York (1954).
18. Kass, E. H., Ingbar, S. H., Harris, J. W., and Ley, A. B., *J. Clin. Invest.*, **30**, 652 (1951).
19. Conn, H. W., Louis, L. H., and Johnston, M. W., *J. Lab. and Clin. Med.*, **34**, 255 (1949).
20. Lazarow, A., *Diabetes*, **1**, 171 (1952).
21. Bichel, J., *Acta med. Scand.*, **117**, 474 (1944).
22. Ling, C. T., and Chow, B. F., *J. Biol. Chem.*, **202**, 445 (1953).
23. Rall, T. W., and Lehninger, A. L., *J. Biol. Chem.*, **194**, 119 (1952).
24. Fegler, G., *Nature*, **170**, 624 (1952).
25. Benesch, R. E., and Benesch, R., *Arch. Biochem. and Biophys.*, **48**, 38 (1954).
26. Labarre, J., *Bull. Acad. roy. Méd. Belgique*, **10**, 291 (1945).

OXIDATION-REDUCTION POTENTIALS OF THE DIPHOSPHOPYRIDINE NUCLEOTIDE SYSTEM*

By F. LEE RODKEY

(From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts)

(Received for publication, July 28, 1954)

Diphosphopyridine nucleotide (DPN) and its reduction product (DPNH) are known to function as a coenzyme system in a variety of reversible biological oxidation-reduction reactions. Despite the demonstrated reversible nature of many enzymatic systems requiring DPN, the coenzyme has not yielded satisfactorily to potentiometric measurement of its oxidation-reduction potential. Several estimates of the oxidation-reduction potential of this system have been made from equilibrium and thermochemical data (9, 6, 4, 18). Values obtained in these studies are widely variable, owing to the technical difficulties of determining the equilibrium constants accurately and the uncertainty in the potential values for some of the substrate systems employed. Recently, a careful equilibrium study by Burton and Wilson (5) has appeared in which some of these difficulties have been overcome.

The first direct measurement of the oxidation-reduction potential of DPN was reported by Ball and Ramsdell (3). The potential was estimated colorimetrically by reduction of oxidation-reduction dyes with DPNH as catalyzed by milk xanthine oxidase. Preliminary data presented from this laboratory (17) were obtained by use of potentiometric techniques.

The present study was undertaken in an attempt to obtain accurate potentiometric data on the DPN system. Reduction of benzyl viologen by DPNH in the presence of certain enzymatic preparations was obtained by Green and Dewan (9). The catalytic effect of milk xanthine oxidase on the reduction of dyes by DPNH has been observed by Ball and Ramsdell (3). Xanthine oxidase and benzyl viologen have therefore been used to obtain reliable potentiometric data on the DPN system over a wide pH range.

EXPERIMENTAL

Apparatus—The jacketted reaction vessels and temperature control procedures have been previously described (16). The reaction vessel was

* This work was supported in part by funds received from the Eugene Higgins Trust and the Ella Sachs Plotz Foundation through Harvard University.

closed with a tight fitting rubber stopper carrying a nitrogen inlet, nitrogen outlet, two bright platinum electrodes, an agar-KCl salt bridge, and a glass tube closed by a vaccine port. The nitrogen inlet extended below the surface of the liquid and the gas served to stir the solution as well as to sweep out the oxygen. The platinum electrodes were rectangular in shape, 1 × 1.5 cm. The vaccine port permitted the introduction of the enzyme and dye solution after partial deoxygenation and also allowed samples of the reaction mixture to be removed for spectrophotometric analysis without addition of oxygen to the cell. Oil-pumped nitrogen (Linde) was freed of oxygen by passing the gas over hot copper (600°) and then through a 5 per cent solution of chromous chloride. Chromous chloride solution was prepared by reducing a 5 per cent solution of chromic chloride with amalgamated zinc and dilute hydrochloric acid. Control experiments were performed in which the amalgamated zinc was removed from the chromous chloride solution and excess hydrogen displaced with a stream of nitrogen. These experiments indicated that the small amount of hydrogen released into the nitrogen stream when the amalgamated zinc was left in contact with the acid solution did not alter the observed oxidation-reduction potential of the DPN system. Hence the chromium salt was conveniently maintained in the reduced state by leaving the amalgamated zinc in contact with the acid solution in a gas-washing bottle. The nitrogen was then bubbled through water containing phenol red and a drop of dilute sodium hydroxide in order to insure the absence of HCl from the gas stream. Potential measurements were made with a Leeds and Northrup No. 7660 potentiometer. Potentials are in absolute volts. A saturated calomel half cell was the reference electrode. This electrode was maintained in a water bath at 30° and standardized at weekly intervals.

Materials—Oxidized DPN was obtained from the Pabst Laboratories. This material assayed 89 per cent DPN by the cyanide procedure of Colowick *et al.* (7) and was used without further purification. Chemically reduced DPNH was prepared by the method of Ohlmeyer (13). Different samples of DPNH prepared from Pabst DPN by this procedure were between 70 and 76 per cent pure as indicated by ultraviolet absorption at 340 mμ. Preparation of DPNH by enzymatic reduction was accomplished by the method of Pullman *et al.* (14) and by the method of Fisher *et al.* (8).

Mediators—Rosinduline (rosinduline 2G, Rowe's "Colour index," No. 830) and benzyl viologen were obtained from The British Drug Houses, Ltd. Alizarin S was obtained from The Coleman and Bell Company, Inc., and safranine T from the National Aniline and Chemical Company, Inc. The commercial dyes were used without further purification. Dye solutions, prepared by dissolving the dye in water immediately before use,

were of such concentration that the addition of 1 ml. of dye solution to the contents of the potentiometric cell gave a final concentration of mediator near 1×10^{-5} M.

Xanthine Oxidase—This enzyme was prepared from cream by the method of Ball (2). The enzyme was kept at 4° under 40 per cent saturated ammonium sulfate. In order to prepare the enzyme for use, the precipitated protein was recovered by centrifugation. The ammonium sulfate supernatant fluid was removed and the precipitate was dissolved in water (0.5 ml. of precipitate per 3 ml. of final enzyme solution). 1 ml. of this enzyme solution was routinely added to each potentiometric cell. Protein nitrogen of the enzyme solution was determined after exhaustive dialysis to remove ammonium sulfate. The dialyzed solution, which yielded a negative test for free ammonia, gave a protein nitrogen content of 0.6 mg. per ml. by micro-Kjeldahl analysis. This corresponds to about 4 mg. of protein per ml. Since the volume of the cell contents was about 20 ml. during the potentiometric measurements, the concentration of protein in the cell was about 0.2 mg. per ml. By use of the approximate molecular weight (200,000) given by Avis *et al.* (1), the final concentration of xanthine oxidase in the solution used for potentiometric measurements was estimated to be about 1×10^{-6} M.

Buffers—Preliminary experiments indicated that DPNH was less stable in neutral or slightly alkaline solutions when phosphate buffers were used. Kaplan *et al.* (12) have reported that the rate of decomposition of DPN in alkaline solutions is markedly increased by the presence of inorganic phosphate. For these reasons, phosphate buffers were not used. Veronal buffers were employed in a number of preliminary experiments at pH 8.0 with satisfactory results. Near pH 7.0, however, it was difficult to prepare Veronal buffers with sufficient buffering capacity because of the relatively low solubility of diethylbarbituric acid. Buffers prepared with tris(hydroxymethyl)aminomethane and glycine were used between pH 7.2 and 9.8. This pH range was extended to more acid and alkaline values with buffers prepared from Versene. Although Versene is not commonly used as a buffer, the third and fourth dissociation constants ($pK_3 = 6.16$, $pK_4 = 10.26$) of ethylenediaminetetraacetic acid (19) are such that solutions of adequate buffering capacity could be prepared near pH 7.0 and 10.5. Buffers prepared from Versene were used in these ranges after it was found that xanthine oxidase showed normal enzymatic activity in the presence of Versene.

All buffers were 0.1 M and pH measurements were performed at the completion of each potentiometric determination. All pH values given were determined by the hydrogen electrode. The calomel half cell was standardized against the hydrogen electrode in 0.1 M acetic acid plus 0.1 M sodium

acetate at 30°. The pH of this solution as interpolated from the data of Hitchcock and Taylor (10, 11) is 4.648.

Procedure

The oxidation-reduction potentials of the DPN system were determined by the method of mixtures and by oxidative titration. A solution of reduced and oxidized DPN was prepared by dissolving the compounds in the desired buffer in such a manner that the total concentration of the two forms was between 1×10^{-4} and 1×10^{-3} M. 15 ml. of this solution were placed in the reaction vessel and deoxygenated for 10 minutes. Xanthine oxidase solution was added from a hypodermic syringe through the vaccine port and deoxygenation continued for an additional 5 minutes. The mediator solution was added and the potential values recorded until equilibrium was established. Equilibrium was assumed when the potential did not change more than 0.5 mv. in a period of 30 to 45 minutes and the values recorded at the two platinum electrodes were less than 0.5 mv. apart. When benzyl viologen was used as a mediator, these criteria were fulfilled about 60 to 90 minutes after addition of the dye.

When equilibrium had been established, a sample of the cell contents was removed with a syringe without opening the cell to oxygen. The sample was appropriately diluted and the DPNH concentration determined by measuring the optical density at 325 and 340 mμ with a Beckman model DU spectrophotometer. This solution was then diluted with an equal volume of 2 M KCN and again read at 325 and 340 mμ. The increase in optical density is due to the formation of the cyanide complex of DPN which has an absorption maximum at 325 mμ. The equilibrium concentrations of DPN and DPNH were calculated from the spectrophotometric data with the extinction coefficients as determined by Colowick *et al.* (7). The spectrophotometric analysis was completed within 5 minutes after removing the sample from the cell because DPNH is slowly oxidized by oxygen in the presence of xanthine oxidase and the dye. After completing the DPN assay, the pH of the cell contents was immediately measured by the hydrogen electrode. Below pH 8.0, measurements of pH on the original buffer solution and on the complete reaction mixture were in essential agreement. This indicated that the presence of the DPN did not seriously alter the pH measurements by this method. At higher pH values there was considerable discrepancy between the pH of the original buffer and the pH of the reaction mixture. This was found to be due to titration of the ammonium ion added in the xanthine oxidase preparation.

A number of potentiometric measurements were performed with enzymatically prepared DPNH. It was necessary to remove the barium from the DPNH-barium salt prepared enzymatically and isolated by the

method of Pullman *et al.* (14). This was accomplished by dissolving the desired amount of the barium salt of DPNH in a small volume of water and passing the solution over a column (10 × 0.8 cm.) of Dowex 50 (30 to 50 mesh) in the sodium form. The column was washed with three 2 ml. portions of water and all eluates from the column were combined. Buffer was added to this solution of reduced DPNH and enough oxidized DPN was dissolved in the solution to give a final concentration of DPN plus DPNH near 5×10^{-4} M. From this point, potential measurements were performed as described above.

Results

Several preliminary experiments were performed in an attempt to measure the potentials of DPN-DPNH mixtures in the presence of dyes but without xanthine oxidase. These experiments indicated that safranine T was the only mediator used which interacted with DPNH at a reasonable rate. The potentials, however, were not stable with time and continued to drift to more negative values over a period of 48 hours or more. The addition of xanthine oxidase to the reaction mixture hastened attainment of equilibrium markedly with all dyes tested, but stable and reproducible potentials were most rapidly obtained when benzyl viologen was present. It appeared that the potentials observed in the presence of safranine T were always 25 to 30 mv. more negative than the values obtained when benzyl viologen, rosinduline, or alizarin S was used as a mediator. Furthermore, the potentials measured with safranine T as a mediator did not respond in a reproducible manner to changes in the per cent reduction of the DPN at constant pH.

Equilibrium potentials could be obtained within 90 minutes after addition of the benzyl viologen. Potentials observed with this system at constant pH were dependent on the relative concentration of DPN and DPNH in the manner given in the equation

$$E_h = E'_0 + 0.0301 \log \frac{[DPN^+]}{[DPNH]} \quad (1)$$

The E'_0 values which were obtained by the method of mixtures are presented in Table I. These calculated E'_0 values do not require correction for the presence of benzyl viologen. This is true because the equilibrium concentrations of both DPN and DPNH were determined in addition to the potential measurement.

Oxidative titration of chemically prepared DPNH was performed at pH 8.37 and 30°. 17 ml. of 1.2×10^{-3} M DPNH (20.4 μmoles) containing twice the usual amount of xanthine oxidase were prepared and deoxygenated. Benzyl viologen was added as a mediator at about 1 per cent of

the equivalent concentration of DPNH (2.5×10^{-5} M) and potential measurements were performed in the usual manner. After equilibrium was obtained, the DPNH was titrated with increments of 0.01 M potassium ferricyanide. Equilibrium potentials were obtained after each addition of oxidizing agent. The rate of oxidation of the DPNH by the $K_3Fe(CN)_6$ was much slower than that of the benzyl viologen. This was shown by the

TABLE I

Oxidation-Reduction Potentials of Diphosphopyridine Nucleotide System Determined by Method of Mixtures at 30°

pH	Buffer	DPN + DPNH, M × 10^5	Per cent reduction	Benzyl viologen, M × 10^5	E'_0	E'_0, calculated*
6.54	Versene	5.59	27.9	2.1	−0.3042	−0.3040
6.64	"	6.40	47.0	2.3	−0.3026	−0.3070
7.17	Tris†	10.25	45.0	2.5	−0.3221	−0.3229
7.35	Versene	8.45	47.1	2.3	−0.3266	−0.3283
7.44	Tris	9.91	38.5	3.2	−0.3326	−0.3310
7.47	"	7.03‡	39.6	2.5	−0.3295	−0.3319
7.94	"	7.05‡	31.0	2.1	−0.3501	−0.3461
8.01	"	9.66	46.6	2.5	−0.3458	−0.3482
8.38	Glycine	8.88‡	51.8	2.5	−0.3666	−0.3593
8.39	Tris	5.29	35.8	2.0	−0.3581	−0.3596
8.97	Glycine	10.19	42.9	2.5	−0.3777	−0.3771
9.34	"	6.67‡	35.8	2.5	−0.3851	−0.3882
9.47	"	8.61	36.2	3.5	−0.3929	−0.3921
9.53	"	7.07‡	21.9	2.1	−0.3991	−0.3940
9.56	Versene	8.76	47.5	2.3	−0.3958	−0.3949
9.84	Glycine	7.51	51.3	2.5	−0.3989	−0.4033
10.08	Versene	4.40	45.0	2.3	−0.4071	−0.4105
10.45	"	3.35	17.1	2.1	−0.4271	−0.4216

* Calculated from the equation $E'_0 = -0.1071 - 0.0301$ pH.

† Tris(hydroxymethyl)aminomethane.

‡ Enzymatically prepared DPNH.

observation that addition of less than 0.05 ml. of oxidizing agent caused complete disappearance of the blue color from the solution. When excess DPNH remained, however, the yellow color of $K_3Fe(CN)_6$ gradually faded as the DPNH was oxidized. Within 1 minute after the yellow color had faded, the blue color of the reduced dye appeared and gradually increased in intensity until equilibrium was attained. This series of color changes was uniformly observed at each addition of $K_3Fe(CN)_6$ until all the DPNH had been oxidized.

At the completion of the titration, the cell contents were assayed for the amount of DPN present. The assay showed that the total DPN present

was 19.7 μmoles, in satisfactory agreement with the amount of DPNH at the beginning of the titration. The amount of $K_3Fe(CN)_6$ required for the titration (41.9 μmoles) indicates that 2 moles of $K_3Fe(CN)_6$ are required for the oxidation of 1 mole of DPNH. The results of the titration and the E'_0 values calculated by the method of Reed and Berkson (15) are given in Table II. The E'_0 of the benzyl viologen (−0.359) is nearly identical with that of the DPN system (−0.361) at this pH. Since the mediator was present at only 1 per cent of the equivalent concentration

TABLE II

Oxidative Titration of DPNH

Temperature = 30°, pH = 8.37, oxidant = $K_3Fe(CN)_6$ (0.01 M), DPNH present initially = 20.4 μmoles, total oxidizing agent for complete titration 4.19 ml. = 41.9 μmoles, DPN present at completion of titration = 19.7 μmoles.

Amount of oxidizing agent added		Per cent oxidation	E_h, observed	E'_0, calculated†	Deviation from average
Total (y)	Corrected ($y - d$)*				
ml.	*ml.*		*volts*	*volts*	*mv.*
0.0	0.440	10.50	−0.3905	−0.3625	−1.0
0.2	0.640	15.27	−0.3835	−0.3611	+0.4
0.4	0.840	20.05	−0.3780	−0.3599	+1.6
1.0	1.440	34.37	−0.3705	−0.3621	−0.6
2.0	2.440	58.25	−0.3575	−0.3618	−0.3
3.0	3.440	82.10	−0.3425	−0.3624	−0.9
3.5	3.940	94.03	−0.3245	−0.3605	+1.0
Average.....				−0.3615	

* ($-d$) represents the ml. of $K_3Fe(CN)_6$ equivalent to the oxidized DPN present before titration was started.

† Calculated from the equation $E_h = E'_0 + 0.0301 \log ([DPN^+]/[DPNH])$.

of the DPN system, no corrections of the E'_0 values owing to the presence of the mediator have been attempted. Such corrections would be beyond the sensitivity of the potentiometric measurements and would not cause appreciable distortion of the titration curve.

The results of this titration are convincing evidence that this system is a reversible system whose potential, at constant pH, is accurately described by the equation

$$E_h = E'_0 + \frac{RT}{nF} \ln \frac{[DPN^+]}{[DPNH]} \qquad (2)$$

The evidence that $n = 2$ is obtained not only from the correlation be-

tween the theoretical curve and the experimental points, but also from the stoichiometry of the titration, indicating that 2 moles of $K_3Fe(CN)_6$ are required per mole of DPNH oxidized.

Results of potential measurements performed with this system at 30° are shown in Fig. 1. The line was determined by the method of least squares and is expressed throughout the pH range investigated by the em-

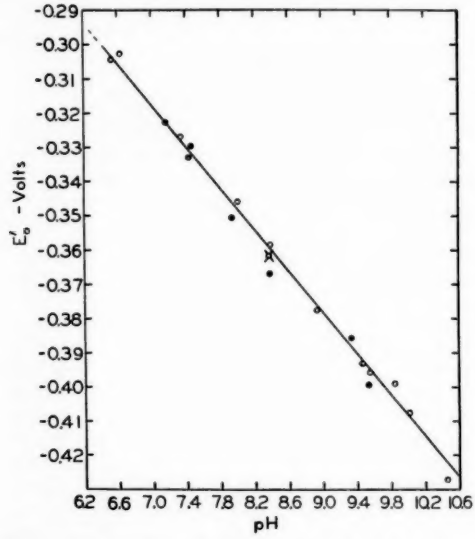


FIG. 1. E'_0-pH curve of the diphosphopyridine nucleotide system. ○, points determined with chemically prepared DPNH; ●, points determined with enzymatically prepared DPNH; ⊠, the result obtained by oxidative titration of chemically prepared DPNH.

pirical equation

$$E'_0 = -0.1054 - 0.0303 \text{ pH} \quad (3)$$

It has not been possible to determine the potentials below pH 6.5 or above 10.5 because of the rapid decomposition of DPN and DPNH in alkaline solution and of DPNH in acid solution. The effect of pH on the potential below 6.5 remains in doubt and the true value of E'_0 at pH 0 uncertain. However, if one uses the average experimental figure for E'_0 at pH 7.0 (−0.3178 volt) and assumes the theoretical change of E'_0 with pH (−0.0301), the value of the intercept at pH 0 is −0.1071 volt and the equation describing the oxidation-reduction potential of the DPN-DPNH

system between pH 6.5 and 10.5 at 30° becomes

$$E_h = -0.1071 + 0.0301 \log \frac{[DPN^+]}{[DPNH]} - 0.0301 \text{ pH} \quad (4)$$

Conclusions

The oxidation-reduction potential of the DPN system at pH 7.0 and 30° reported here (−0.318 ± 0.003 volt) is in essential agreement with the value calculated from equilibrium data (−0.320 ± 0.004 volt at 25°) by Burton and Wilson (5). A limited number of potential measurements have been performed at different temperatures. From these data, a preliminary estimate of the temperature coefficient ($\Delta E'_0/\Delta T = -0.001$ volt per degree) for this system has been obtained. By use of this preliminary value for the temperature coefficient, the result of Burton and Wilson at 25° would become −0.325 ± 0.004 volt at 30°, a value approximately 7 mv. more negative than the one determined potentiometrically.

These results do not agree with the preliminary titration data reported by Rodkey and Ball (17). In these earlier titrations, however, it was not possible to measure the actual concentrations of DPN and DPNH with accuracy. Furthermore, these earlier experiments were all performed in phosphate buffers between pH 6.8 and 8.0 over long periods of time. As the present work proceeded, it became apparent that these conditions favor the decomposition of DPNH at an appreciable rate.

The E'_0-pH curve presented for the DPN system closely approaches that showing the theoretical slope ($\Delta E'_0/\Delta\text{pH} = -0.0301$ volt per pH) to be expected if the oxidation-reduction reaction proceeds according to the equation

$$DPNH \rightleftharpoons DPN^+ + 2e + H^+ \quad (5)$$

The slope of this curve does not change between pH 6.5 and 10.5, indicating that any proton dissociations must be similar in both the oxidant and reductant throughout this range. Rapid decomposition of the oxidant or reductant outside this pH range made it impractical to attempt potential measurements over wider limits. This decomposition is not caused by the xanthine oxidase preparation, since similar rates of destruction are observed in the absence of the enzyme.

Potentiometric measurements with DPNH prepared by chemical or enzymatic methods yield similar results within the limits of the present method. It therefore seems unlikely that the variation of the potentials of this system recorded in the literature can result from the mode of reduction of the coenzyme. It has been suggested (20) that the oxidation-reduction potential of the free coenzyme is different from the potential observed in the presence of a specific DPN-linked enzyme, liver alcohol dehydrogen-

ase. It has been shown by spectrophotometric means that horse liver alcohol dehydrogenase has an affinity for the coenzyme which is greater for DPNH than for DPN (21). Such differences in the affinity of the enzyme for the coenzyme should be reflected in an alteration of the E'_0 measured and would be influenced by the amount of enzyme present. In the present experiments a 4-fold increase in the amount of xanthine oxidase did not change the measured E'_0. This is not surprising, since the total molar concentration of the coenzyme was approximately 1000 times as great as the concentration of protein. Under these conditions, any complex formation between the enzyme and coenzyme would be expected to cause only minor variations in the measured potentials.

SUMMARY

Oxidation-reduction potentials of the diphosphopyridine nucleotide system have been determined potentiometrically at 30° from pH 6.5 to 10.5. The E'_0 of this system at pH 7.00 was found to be -0.318 volt and the slope of the E'_0-pH curve is -0.0301 volt per pH throughout the range investigated.

The author wishes to express his deep appreciation to Dr. Eric G. Ball for his continued interest and constant advice throughout the progress of this investigation.

BIBLIOGRAPHY

1. Avis, P. G., Bergel, F., Bray, R. C., and Shooter, K. V., *Nature*, **173**, 1230 (1954).
2. Ball, E. G., *J. Biol. Chem.*, **128**, 51 (1939).
3. Ball, E. G., and Ramsdell, P. A., *J. Biol. Chem.*, **131**, 767 (1939).
4. Borsook, H., *J. Biol. Chem.*, **133**, 629 (1940).
5. Burton, K., and Wilson, T. H., *Biochem. J.*, **54**, 86 (1953).
6. Clark, W. M., *J. Appl. Phys.*, **9**, 97 (1938).
7. Colowick, S. P., Kaplan, N. D., and Ciotti, M. M., *J. Biol. Chem.*, **191**, 447 (1951).
8. Fisher, H. F., Conn, E. E., Vennesland, B., and Westheimer, F. H., *J. Biol. Chem.*, **202**, 687 (1953).
9. Green, D. E., and Dewan, J. C., *Biochem. J.*, **31**, 1069 (1937).
10. Hitchcock, D. I., and Taylor, A. C., *J. Am. Chem. Soc.*, **59**, 1812 (1937).
11. Hitchcock, D. I., and Taylor, A. C., *J. Am. Chem. Soc.*, **60**, 2710 (1938).
12. Kaplan, N. O., Colowick, S. P., and Barnes, C. C., *J. Biol. Chem.*, **191**, 461 (1951).
13. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
14. Pullman, M. E., Colowick, S. P., and Kaplan, N. O., *J. Biol. Chem.*, **194**, 593 (1952).
15. Reed, L. J., and Berkson, J., *J. Phys. Chem.*, **33**, 760 (1929).
16. Rodkey, F. L., and Ball, E. G., *J. Biol. Chem.*, **182**, 17 (1950).
17. Rodkey, F. L., and Ball, E. G., *Proc. Nat. Acad. Sc.*, **38**, 396 (1952).
18. Schlenk, F., Hellström, H., and von Euler, H., *Ber. chem. Ges.*, **71**, 1471 (1938).
19. Schwarzenbach, G., and Ackermann, H., *Helv. chim. acta*, **31**, 1029 (1948).
20. Theorell, H., and Bonnichsen, R., *Acta chem. Scand.*, **5**, 329 (1951).
21. Theorell, H., and Bonnichsen, R., *Acta chem. Scand.*, **5**, 1105 (1951).

XUM

AROMATIC BIOSYNTHESIS

XIV. 5-DEHYDROSHIKIMIC REDUCTASE

By HAIM YANIV* AND CHARLES GILVARG†

(From the United States Public Health Service, Tuberculosis Research Laboratory, Cornell University Medical College, New York, New York)

(Received for publication, July 27, 1954)

Shikimic acid, a 3,4,5-trihydroxycyclohexene-1-carboxylic acid, has been shown to be a precursor of tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid in *Escherichia coli* and other bacterial species (1, 2). Two additional precursors of these aromatic compounds are accumulated by certain mutants and have been identified as 5-dehydroquinic acid (DHQ)[1] (3) and 5-dehydroshikimic acid (DHS) (4). On the basis of accumulations and nutritional responses of bacterial mutants blocked in different reactions on the aromatic pathway, it could be concluded that the biosynthetic sequence proceeds from DHQ to DHS to shikimic acid (5).

Extracts prepared from *E. coli* and other sources have been found to catalyze the conversion of DHQ to DHS, and this reaction has been shown to be due to a specific dehydrase, 5-dehydroquinase (6). In the present work similar extracts have been found to catalyze the conversion of DHS to shikimic acid, and evidence will be presented that the reaction is due to a specific TPN-linked reductase. Some properties of the partially purified enzyme will be described. It is proposed that this enzyme be called 5-dehydroshikimic reductase.

EXPERIMENTAL

The reaction studied is shown in the accompanying scheme.

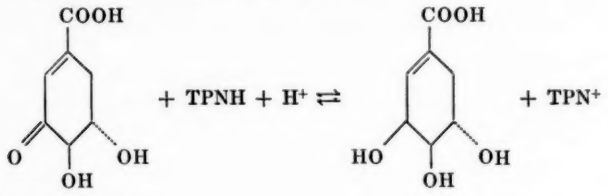
Spectrophotometric Assay—Although the net reaction in the direction of shikimic acid is the one that takes place biosynthetically, and the one favored by the equilibrium at pH 7 or 8, DHS and TPNH are less readily

* Fellow of the Israeli Council of Science. Deceased, April 29, 1954.

† Fellow of The National Foundation for Infantile Paralysis, Inc. Present address, Department of Biochemistry, New York University College of Medicine, New York 16.

[1] The following abbreviations are used: DHQ, 5-dehydroquinic acid; DHS, 5-dehydroshikimic acid; GSSG and GSH, oxidized and reduced glutathione; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; SH, sulfhydryl groups; Tris, tris(hydroxymethyl)aminomethane.

available reagents than are shikimic acid and TPN^+. It was therefore more convenient to follow the reverse reaction.



5-Dehydroshikimic acid — Shikimic acid

Extracts prepared from *E. coli* were found to catalyze the oxidation of shikimic acid by TPN and the reconversion of the resulting TPNH to TPN upon addition of DHS (Fig. 1). The reaction was followed by measuring the increment in optical density at 340 mμ resulting from the conversion of TPN^+ to TPNH.

Assay by Coupling with GSH Formation—The unfavorable equilibrium limits the range of linearity of the above assay with respect to enzyme concentration. A more satisfactory assay was therefore developed by coupling the reaction (A) with the essentially irreversible (7) action of GSSG reductase (B), giving the over-all reaction (C).

(A) Shikimic acid + $TPN^+ \rightleftharpoons$ DHS + TPNH + H^+
(B) TPNH + H^+ + GSSG → TPN^+ + 2GSH

(C) Shikimic acid + GSSG → DHS + 2GSH

The over-all reaction requires that for each mole of shikimic acid which disappears 1 mole of DHS and 2 moles of GSH appear. As can be seen from Table I, this stoichiometry is closely approached.

In the presence of an excess of GSSG reductase, the DHS reductase activity becomes rate-limiting and hence can be assayed through the determination of SH released. The initial extracts prepared from *E. coli* are rich in GSSG reductase, but this activity is lost upon fractionation. An exogenous source of the enzyme was therefore routinely added in experiments involving the above coupled reaction. Guinea pig liver extract prepared according to Rall and Lehninger (7) was used for this purpose. Since some preparations of guinea pig liver extract released SH from GSSG, a control assay in which shikimic acid was omitted was performed for each determination. Ethylenediaminetetraacetate was also added to the reaction mixture in order to prevent SH loss, due to metal-catalyzed oxidation by air, and SH formation by metal-requiring enzyme reactions. Under these conditions the amount of SH formed is proportional to the

concentration of DHS reductase (Fig. 2). The purification of the enzyme was carried out with the aid of this assay.

Definition of Unit—A unit of enzyme is defined as the amount that catalyzes the formation of 1 μmole of DHS per minute under the condi-

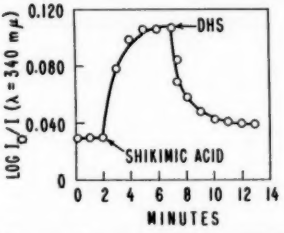


FIG. 1. Reversibility of the reaction catalyzed by DHS reductase. 200 μmoles of Tris buffer, pH 8.0; 0.130 μmole of TPN; initial extract of *Escherichia coli* 4.6 mg. of protein. Final volume made up to 3.0 ml. with water. Later additions 0.10 μmole of shikimic acid and 0.50 μmole of DHS. Silica cells 1.0 cm. light path; temperature 30°.

TABLE I

Stoichiometry of Over-All Reaction

The reaction mixture contained 200 μmoles of Tris buffer, pH 8.0; 20 μmoles of ethylenediaminetetraacetate, pH 8.0; 0.20 μmole of TPN; 3.00 μmoles of sodium shikimate; 5 μmoles of GSSG; guinea pig liver extract 0.20 ml.; *E. coli* initial extract 9.1 mg. of protein. Final volume 3.0 ml. Incubated at 30° for 60 minutes.

Shikimic acid*	DHS†	Glutathione‡
Δ μmoles	Δ μmoles	Δ μmoles
−3.0	+2.9	+5.8

* Bioassay with mutant 83-2 (5), which responds to shikimic acid but not to DHS, indicated that at least 99.5 per cent of the shikimic acid was transformed during the reaction.

† Determined by the change in absorption at 234 mμ. DHS has an absorption maximum at this wave-length; ϵ_{234} in water = 11,900 (4). The material formed was qualitatively identified by means of bacterial mutant 83-1 (5), which responds to DHS or shikimic acid but not to DHQ.

‡ Determined as change in sulfhydryl.

tions of the assay. Specific activity is expressed as units per mg. of protein.

Purification—E. coli (strain W) was grown with aeration on Medium A (8) plus 0.2 per cent Difco yeast extract, harvested after 24 hours, and lyophilized. All subsequent operations were carried out at 0°.

Initial extracts were prepared by grinding the lyophilized cells with 3 times their weight of Pyrex glass powder for 5 minutes and then for an

additional 10 minutes with gradual additions of M/30 Tris buffer, pH 8.0 (20 ml. per gm. dry weight of bacteria). Centrifugation for 20 minutes at 5000 × *g* gave a clear yellow supernatant fluid which was adjusted to a protein concentration of 20 mg. per ml. In order to reduce the nucleic acid content of the extract, one-twentieth its volume of 1 M $MnCl_2$ was added over a period of 10 minutes with occasional stirring (9). A volumi-

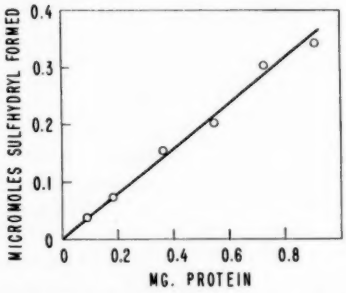


FIG. 2. Sulfhydryl production as a function of enzyme concentration. 40 μmoles of Tris buffer, pH 8.0; 4 μmoles of ethylenediaminetetraacetic acid; 1 μmole of GSSG; 1 μmole of sodium shikimate; 0.04 μmole of TPN; 0.04 ml. of guinea pig liver extract; *E. coli* initial extract; final volume 0.60 ml.; incubated at 30° for 20 minutes. Reaction stopped by addition of 0.60 ml. of 6 per cent metaphosphoric acid.

TABLE II

Purification of DHS Reductase

Step	Protein concentration	Specific activity	Total activity	Yield
	mg. per ml.	*units per mg.*	*units*	*per cent*
Initial extract	20	0.0082	7.9	100
$MnCl_2$ supernatant	8.4	0.0183	6.0	76
Calcium phosphate gel eluate	2.2	0.025	2.21	28
Ammonium sulfate fraction 0.32–0.45	2.9	0.078	1.18	15

nous precipitate was obtained and was removed by centrifuging at 5000 × *g* for 20 minutes.

After adjusting the $MnCl_2$ supernatant fluid to pH 5.5 with 0.1 M acetic acid, 0.2 volume of calcium phosphate gel was added and the whole was stirred for 20 minutes and then centrifuged. The precipitated gel was discarded. To the supernatant fluid 1.2 volumes of gel were added, and the procedure was repeated except that the gel was retained. It was washed by suspension in several volumes of water and centrifugation. The washed gel was eluted by being stirred for 20 minutes in a volume of

0.1 M phosphate buffer, pH 6.0, equal to the volume of the $MnCl_2$ supernatant fluid. The eluted gel was removed by centrifugation.

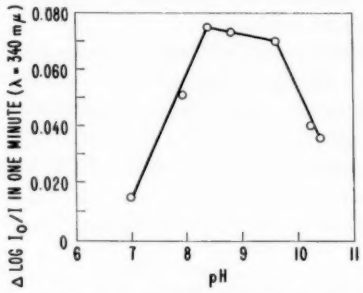


FIG. 3. pH optimum. 200 μmoles of buffer (phosphate, Tris, or carbonate); 1 μmole of shikimic acid; 0.13 μmole of TPN; purified enzyme 0.29 mg. of protein. Final volume made up to 3.0 ml. with water. Silica cells 1.0 cm. light path; temperature 30°. pH measured at the end of the reaction with a glass electrode.

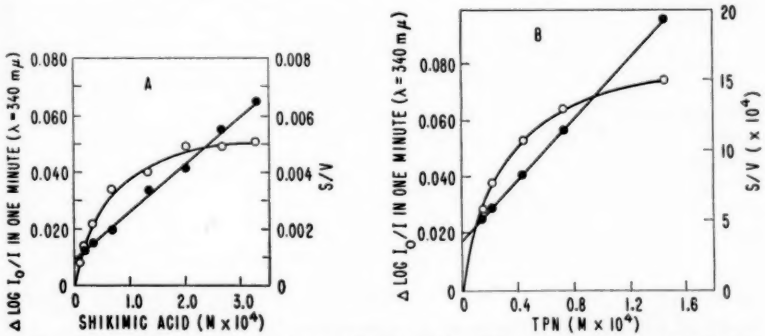


FIG. 4. *A*, Michaelis constant for shikimic acid. 200 μmoles of Tris, pH 8.0; 0.13 μmole of TPN; purified enzyme 0.29 mg. Final volume 3.0 ml. Silica cells 1.0 cm. light path; temperature 30°. *B*, Michaelis constant for TPN. 200 μmoles of Tris buffer, pH 8.0; 5 μmoles of shikimic acid; purified enzyme 0.40 mg. Final volume 3.0 ml. Silica cells 1.0 cm. light path; temperature 30°. The left-hand ordinate refers to the change in optical density at 340 mμ observed in the 1st minute of reaction (○), and the right-hand ordinate scale refers to a Lineweaver-Burk plot of substrate concentration divided by velocity (S/V) as a function of substrate concentration (●).

The supernatant fluid was subjected to ammonium sulfate fractionation at pH 7.0. The material precipitating between 32 and 45 per cent saturation was dissolved in M/30 Tris buffer, pH 8.0, and dialyzed overnight against the same buffer. Table II summarizes a typical fractionation.

The product had been freed of the enzyme that forms DHS from DHQ. As the enzymatic step subsequent to shikimic acid on the aromatic pathway is at present unknown, the presence or absence of the corresponding enzyme could not be established.

The enzyme at this stage of purification retained 80 per cent of its activity after storage at −15° for 4 months.

pH Optimum—Since the GSSG reductase of guinea pig liver was found to be rapidly inactivated at pH values above 8.5, the coupled reaction was not suitable for measuring the pH optimum of DHS reductase. Therefore the spectrophotometric method of assay was utilized, the change in optical density at 340 mμ obtained in the first minute of reaction being measured

TABLE III

Specificity of DHS Reductase

Each compound was tested at 0.0017 M as a substrate for DHS reductase. The conditions were those described in Fig. 2 except that purified enzyme was used (50 γ per tube, specific activity 0.10). Each compound was also tested at 0.005 M as an inhibitor of DHS reductase, acting on shikimic acid at 0.0017 M; 75 γ of purified enzyme per tube.

Compound	Activity as substrate	Activity as inhibitor
	per cent of shikimic acid	*per cent inhibition*
Shikimic acid	100	
3-Phosphoshikimic acid	<3	−3
5-Phosphoshikimic "	<3	8
5-Epishikimic acid	<3	47
Dihydroepishikimic acid	<3	29
Quinic acid	<3	14

at various pH values. The enzyme was found to be active over a broad range of pH, with the optimum at approximately 8.5 (Fig. 3).

Michaelis Constants—The Michaelis constants for shikimic acid and TPN were determined at pH 8.0 by following the initial rates of TPN reduction in the spectrophotometer. Michaelis constants of 5.5×10^{-5} M and 3.1×10^{-5} M were obtained for shikimic acid and TPN respectively (Figs. 4, *A*, and 4, *B*).

Equilibrium Constant—The reaction between TPN and shikimic acid was allowed to proceed to equilibrium in the spectrophotometer. TPNH concentration was determined at 340 mμ and DHS concentration (4) at 234 mμ. The two values were in close agreement, as is required from the stoichiometry of the reaction. The resulting values calculated for K [H⁺] at 30° were 5.7 at pH 7.9 and 27.7 at pH 7.0

$$K\,[H^+] = \frac{[\text{shikimic}][TPN^+]}{[DHS][TPNH]}$$

Specificity—DPN could not replace TPN as electron acceptor with the purified enzyme. A variety of substances structurally related to shikimic acid were tested as possible substrates of DHS reductase and found to be without activity (Table III).

Distribution—Extracts were prepared from several microorganisms, plants, and animal tissues. The results are presented in Table IV. The enzyme was found to be present only in those organisms capable of synthesizing the aromatic amino acids. Of particular interest is the absence of the enzyme from *E. coli* mutants 83-2 and 156-53, which accumulated

TABLE IV

Distribution of DHS Reductase

Unless otherwise stated the enzyme extracts were prepared by grinding material with glass powder. The assays were performed according to the conditions described in Fig. 2.

Organism	Specific activity, μmoles per min. per mg. protein × 10^3
Escherichia coli (wild type strain 9637 ATCC)	8.2
Aerobacter aerogenes (wild type)	9.0
E. coli 83-2	0 (<0.1)
" " 156-53*	0 (<0.1)
" " 83-1*	5.0
Euglena gracilis	3.2
Saccharomyces cerevisiae	2.9
Spinach†	1.8
Peas†	5.6
Guinea pig (liver)‡	0 (<0.1)

* Activity measured in a Beckman spectrophotometer.

† Enzyme extract prepared in a Waring blendor.

‡ Water extract of acetone powder.

DHS and therefore were presumed to be blocked between DHS and shikimic acid. *E. coli* mutant 83-1, which is blocked before DHS, yielded the same amount of DHS reductase as the wild type.

DISCUSSION

Extracts of wild type *E. coli* have been found to contain a specific TPN-linked enzyme, DHS reductase, capable of interconverting DHS and shikimic acid, whereas this enzyme could not be detected in extracts of a mutant whose block, as inferred from nutritional studies, is located between these two compounds. The lack of a demonstrable cofactor requirement (other than TPN) and the resemblance of the reaction to other dehydrogenations furnish evidence that a single enzyme is involved. The low Michaelis constant for shikimic acid and the substrate specificity demon-

strated in Table III suggest that DHS and shikimic acid are the physiological substrates of this enzyme.

These observations lend considerable support to a scheme (5) that proposes DHQ, DHS, and shikimic acid as intermediates in the biosynthesis of a group of aromatic amino acids and vitamins. The growth factor activity of shikimic acid for a *Neurospora* mutant (10) and the presence of traces of this compound in a variety of plants[2] have previously suggested that this metabolic pathway is ubiquitous in organisms capable of synthesizing their aromatic amino acids. The wide-spread distribution of DHS reductase here observed (bacteria, yeast, and plants) and its absence from animal tissue provide further support for this conclusion.

Materials and Methods

TPN of 80 per cent purity and DPN of 90 per cent purity were purchased from the Sigma Chemical Company.

5-Epishikimic acid and dihydroshikimic acid were the gifts of Dr. I. I. Salamon.

3-Phosphoshikimic acid and 5-phosphoshikimic acid were obtained through the generosity of Dr. U. Weiss.

A large culture of *Euglena gracilis* was the gift of Dr. S. Hutner. We are greatly indebted to Dr. W. E. Brown of E. R. Squibb and Sons who supervised the production of a large amount of lyophilized *E. coli*.

Pyrex glass powder, 325 mesh, was obtained from the Corning Glass Works.

Calcium phosphate gel was prepared according to the method of Keilin and Hartree (11).

Sulfhydryl was determined according to the method of Grunert and Phillips (12).

Protein concentration was determined spectrophotometrically according to the method of Warburg and Christian (13).

SUMMARY

5-Dehydroshikimic reductase, an enzyme that catalyzes the TPN-linked reduction of 5-dehydroshikimic acid to shikimic acid, has been detected in extracts prepared from a variety of organisms. The enzyme from *Escherichia coli* has been partially purified. It has a pH optimum of 8.5 and Michaelis constants of 3.1×10^{-5} and 5.5×10^{-5} for TPN and shikimic acid, respectively; it is apparently specific for this cofactor and this substrate.

[2] Personal communication from Dr. B. D. Davis.

XUM

The authors wish to acknowledge the aid and encouragement of Dr. B. D. Davis.

BIBLIOGRAPHY

1. Davis, B. D., *Experientia*, **6**, 41 (1950).
2. Davis, B. D., *J. Biol. Chem.*, **191**, 315 (1951).
3. Weiss, U., Davis, B. D., and Mingioli, E. S., *J. Am. Chem. Soc.*, **75**, 5572 (1953).
4. Salamon, I. I., and Davis, B. D., *J. Am. Chem. Soc.*, **75**, 5567 (1953).
5. Davis, B. D., *J. Bact.*, **64**, 729 (1952).
6. Mitsuhashi, S., and Davis, B. D., *Biochim. et biophys. acta*, in press.
7. Rall, T. W., and Lehninger, A. L., *J. Biol. Chem.*, **194**, 119 (1952).
8. Davis, B. D., and Mingioli, E. S., *J. Bact.*, **60**, 17 (1950).
9. Korkes, S., del Campillo, A., Gunsalus, I. C., and Ochoa, S., *J. Biol. Chem.*, **193**, 721 (1951).
10. Tatum, E. L., Gross, S. R., Ehrensvärd, G., and Garnjobst, L., *Proc. Nat. Acad. Sc.*, **40**, 271 (1954).
11. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **124**, 397 (1938).
12. Grunert, R. R., and Phillips, P. H., *Arch. Biochem.*, **30**, 217 (1951).
13. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1941).

STUDIES ON PHENYLKETONURIA

II. THE EXCRETION OF *o*-HYDROXYPHENYLACETIC ACID IN PHENYLKETONURIA*

By MARVIN D. ARMSTRONG, KENNETH N. F. SHAW, AND KATHRYN S. ROBINSON

(From the Laboratory for the Study of Hereditary and Metabolic Disorders and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City, and the Utah State Training School, American Fork, Utah)

(Received for publication, October 4, 1954)

Phenylketonuria (phenylpyruvic oligophrenia) is an inherited condition in the human in which the ability of the liver to oxidize phenylalanine to tyrosine is impaired (2). Because this pathway represents the major, although not the only, manner in which phenylalanine is metabolized in mammals, considerable amounts of phenylalanine accumulate in the blood and tissues of affected individuals, and phenylalanine and abnormal metabolites derived from it are excreted in the urine. These metabolites are phenylpyruvic acid (3), β-phenyl-L-lactic acid (3), and phenylacetylglutamine (4), the conjugated form in which phenylacetic acid is excreted by the human. In addition to the biochemical abnormalities, most of the individuals who inherit the biochemical defect suffer a profound impairment of mental functioning. It has not been possible up to the present time to establish a connection between the biochemical abnormality and the development of the mental defect.

Experiments in which the phenylalanine intake of phenylketonuric children was restricted until normal blood levels of phenylalanine were obtained and phenylpyruvic acid excretion ceased were carried out in this laboratory (5). The results indicated that the mental defect does not occur as the result of a simple interference with the functioning of an otherwise normal central nervous system, but many observations were made which were in agreement with the idea that toxic metabolites might be responsible for some of the mental symptoms; similar results have been reported by Bickel, Gerrard, and Hickmans (6). In the course of the metabolic experiments, qualitative reactions were obtained with phenylketonuric urine which were not observed in the urine of normal individuals. Ex-

* This work was supported by research grants from the National Institutes of Health, United States Public Health Service. A preliminary report was presented at the meetings of the American Society of Biological Chemists, Atlantic City, April, 1954 (1).

amination of the free organic acids of phenylketonuric urine showed that abnormal indole derivatives are excreted (7) and that some phenolic acids which are present in normal urine in small amounts occur in phenylketonuric urine in greatly increased amounts. The most prominent of these abnormal phenolic compounds has been isolated and has been identified as *o*-hydroxyphenylacetic acid, in confirmation of the initial chromatographic identification recently reported by Boscott and Bickel in a preliminary communication (8). It appears probable that the formation of *o*-hydroxyphenylacetic acid is not directly related to the development of the mental defect.

EXPERIMENTAL

o-Hydroxyphenylacetic acid was prepared by a modification of methods described in the literature (9, 10); the properties of the synthetic material compared favorably with those reported earlier. Phenylalanine was determined in fasting serum by the modification of the method of Kapeller-Adler described earlier (5).

o-Hydroxyphenylacetic acid (*o*-HPAA) may be detected by direct paper chromatography of urine of patients with phenylketonuria; it is visualized by spraying chromatograms with an alkaline solution of diazotized sulfanilic acid. It is more easily distinguished, however, if it is extracted from urine into an organic solvent before chromatography. Urine was acidified to pH 2 and extracted three times with 0.5 volume portions of ethyl acetate; the acids were taken into a small portion of saturated $NaHCO_3$ and then returned to a measured volume of ethyl acetate after acidification. Aliquots containing 2 to 6 γ of *o*-HPAA were chromatographed, and the intensity of the orange dye formed by coupling with diazotized sulfanilic acid was compared with the color produced by chromatography of known amounts of *o*-HPAA. Recovery experiments made by extracting urine to which had been added known amounts of *o*-HPAA were accurate to ± 10 per cent by this procedure. The most satisfactory solvent system for the chromatography of *o*-HPAA has proved to be isopropyl alcohol–aqueous ammonia–H_2O = 8:1:1; $R_F = 0.73$, ascending chromatogram on Whatman No. 1 paper.

Isolation of o-Hydroxyphenylacetic Acid from Phenylketonuric Urine—40 liters of urine (containing 30 gm. of creatinine) from a patient with phenylketonuria were acidified to pH 2 and extracted in portions with 3 × 0.5 volume portions of ethyl acetate. The pooled ethyl acetate solutions were extracted with small portions of saturated $NaHCO_3$ solution to separate the organic acids, and the combined bicarbonate extracts were acidified and extracted with smaller volumes of ethyl acetate. The organic acids were finally obtained in 1 liter of ethyl acetate; this extract contained approximately 2.4 gm. of *o*-HPAA, as estimated by paper chromatography.

XUM

The organic acids in this solution were subjected to a modified countercurrent distribution procedure in which six 1 liter portions of 1.0 M citrate buffer, pH 3.60, were passed through eight 1 liter portions of ethyl acetate. Most of the *o*-HPAA remained in the first portion of ethyl acetate, but a small amount passed into the second and third. The first portion was then subjected to another similar distribution with buffer of pH 3.6. The acids in the combined second and third ethyl acetate portions from both distributions were extracted into a small portion of $NaHCO_3$ solution and saved. The first ethyl acetate portion remaining after the second treatment was then subjected to a similar procedure in which six 1 liter portions of 1.0 M citrate buffer of pH 5.20 were passed through eight portions of ethyl acetate. With this system, most of the *o*-HPAA distributed in Portions 1 to 8, the highest concentration occurring in Portions 2 and 3. Portion 1 was again treated with buffer at pH 5.2, Portions 2 to 8 from both distributions were pooled, and the acids were extracted into a small volume of $NaHCO_3$ solution and combined with Portions 2 and 3 from the distributions at pH 3.6. The combined bicarbonate solutions were acidified to pH 2, saturated with salt, and extracted thoroughly with ethyl acetate, to give a total volume of 150 ml. This solution was again treated by passage of 6 × 150 ml. portions of buffer of pH 5.2 through 8 × 150 ml. portions of ethyl acetate. Portions 2 to 8 were pooled, and the acids were extracted into bicarbonate and then back into a small volume of ethyl acetate. This solution contained approximately 1.0 gm. of *o*-HPAA.

The ethyl acetate was concentrated to a low volume by evaporation at room temperature under a stream of nitrogen. The thick syrup was allowed to stand overnight at room temperature, and was filtered to remove the β-phenyl-L-lactic acid that crystallized. The phenyllactic acid was washed on the filter with a small portion of cold ethyl acetate, the ethyl acetate was again blown off the combined filtrate and washings, and another crop of phenyllactic acid was collected. This procedure was repeated until no more phenyllactic acid crystallized. A total of 2.3 gm. of crude phenyl-L-lactic acid was obtained. This material was recrystallized from hot water (Norit) to yield a nitrogen-free product melting at 122–124°, $[\alpha]_D^{24}$ −21.4° (*c* 1, H_2O) (phenyl-L-lactic acid, m.p. 124°, $[\alpha]_D$ −20.4° (*c* 1.2, H_2O), (11)).

The oil that remained after the removal of the phenyllactic acid was dissolved in 30 ml. of 50 per cent acetic acid, and the solution was clarified with a small amount of Norit and treated with 15 ml. of 10 per cent mercuric acetate in 50 per cent acetic acid. The precipitate, which contained the mercuric complexes of indole acids contaminated with small amounts of phenolic acids, was separated and worked up for indole compounds; the identification of β-3-indolyllactic acid as the major component of this fraction has been reported elsewhere (7). The acetic acid solution was di-

luted with 10 volumes of water, the aqueous solution was extracted exhaustively with ethyl acetate, and the acids were then extracted into a small volume of bicarbonate and finally returned to ethyl acetate. The ethyl acetate was evaporated in a stream of nitrogen to yield a semicrystalline residue; this was suspended in benzene and filtered, and the crystalline precipitate was washed with benzene. This material (390 mg.) had the chromatographic behavior and qualitative reactions of one of

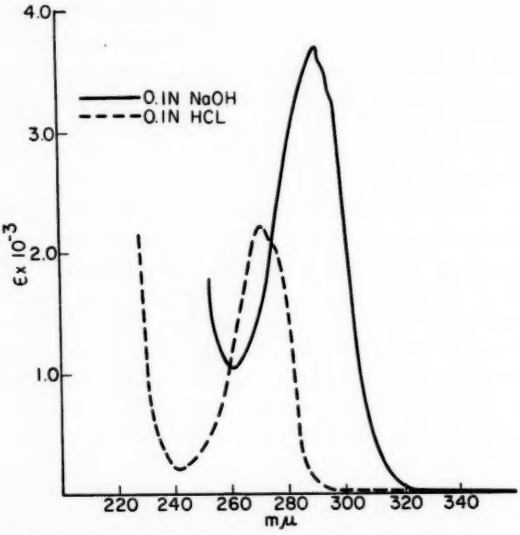


FIG. 1. Absorption spectra of *o*-hydroxyphenylacetic acid

the polar phenolic constituents of normal urine; the characterization and identification of this material will be presented elsewhere.

The residue from the benzene filtrate was dissolved in 50 ml. of 6 N HCl, and the HCl was removed by distillation *in vacuo* at 80°. Crystals of coumaran-2-one, the lactone of *o*-HPAA, began to condense on the walls of the condenser as the last of the acid was removed; the residue had at this time a strong odor of coumaran-2-one. The residue from the distillation was dissolved in ether, and the acids were extracted into bicarbonate. The ether solution of neutral substances was evaporated to dryness, the residue was treated with a small amount of 1 N NaOH to hydrolyze the coumaran-2-one, and the *o*-HPAA formed by this treatment was extracted from the acidified solution with ethyl acetate. The 14 mg. of crude product that was obtained were recrystallized from chlorobenzene to yield 10 mg.

of pure *o*-HPAA. This material had physical and chemical properties identical with those of authentic *o*-HPAA; m.p. 144–145° (authentic 144–146° (10); no depression of the melting point upon admixture with the isolated material). Both authentic and isolated materials had the same crystal form when crystallized from chlorobenzene and showed the same behavior on the micro melting point stage of subliming to form a mixture

TABLE I

Excretion of o-Hydroxyphenylacetic Acid by Phenylketonuric Patients

Name	Age	Intelligence quotient	Fasting serum phenylalanine	Urinary *o*-hydroxyphenylacetic acid
	yrs.		*mg. per 100 ml.*	*γ per mg. creatinine*
Clifford W.	48	70	22	80
Dean D.	18	22	37	100
David R.	14	3	38	175
James A.	11	9	37	135
Linda B.	9	22	40	210
Gary L.	9	40	40	250
Judy D.	8	14	40	235
Allan D.	6	3	38	130
Ann L.	6	3	44	130
David J.	3	50	50	250
Jill D.	3	63	40	300
Robert D.*	1		63	400
" "			17	48
" "			6	3
" "			4	2
" "			2	<1
Mary J.*	1		52	300
" "			10	12
" "			2	<1

* The first value listed for each of these infants was obtained under natural conditions. They were given a diet deficient in phenylalanine (5); the succeeding values were measured at times when the blood phenylalanine was lowered.

of chunky crystals and long prisms just before the melting point. Both materials gave a violet color when a dilute aqueous solution was treated with dilute $FeCl_3$. The chromatographic behavior and color reactions of the two were identical, as were the ultraviolet absorption spectra in dilute acid and dilute alkali (Fig. 1).

The bicarbonate solution obtained in purifying the coumaran-2-one was acidified, and the acids were extracted into ethyl acetate. An additional amount (160 mg.) of crude phenyllactic acid was obtained when this solution was concentrated. The filtrate from the phenyllactic acid was again concentrated to a low volume. A different crystalline compound sep-

arated; this was suspended in benzene, collected on a filter, and washed with benzene. This substance (145 mg.) was impure *o*-HPAA. Ultraviolet spectrophotometry and paper chromatography showed it to contain approximately 65 per cent of *o*-HPAA contaminated with about 4 per cent of *p*-hydroxyphenylacetic acid and 30 per cent of phenyllactic acid. This material was not purified further.

Excretion of o-Hydroxyphenylacetic Acid by Phenylketonurics—Samples of urine from seventeen patients with phenylketonuria have been examined; all showed the excretion of relatively large amounts of *o*-HPAA. The rate of excretion of *o*-HPAA by these patients has been expressed conveniently with respect to the creatinine content of spot collections of urine; the close agreement which has been observed among different samples obtained from a given individual at different times of collection has indicated that this represents a satisfactory measure of the excretion and provides an excellent means for comparing the excretion of individuals of different ages and sizes and with differing degrees of food intake, physical activity, and mental impairment. Table I lists semiquantitative approximations of the rate of excretion of *o*-HPAA by several patients with phenylketonuria.

Excretion of o-Hydroxyphenylacetic Acid by Normal Individuals—All of the normal individuals so far investigated have been found to excrete small, but definite amounts of *o*-HPAA. The amount excreted has been estimated to be 0.3 to 0.5 per mg. of creatinine; for a normal adult, this would amount to about 1 mg. of *o*-HPAA daily.

DISCUSSION

o-Hydroxyphenylacetic acid has been identified as the abnormal phenolic metabolite present in greatest amount in the urine of patients with phenylketonuria. This was accomplished by isolation of the metabolite and by a direct comparison of the properties of the isolated material and synthetic *o*-HPAA. It should be noted that the poor recovery of *o*-HPAA in the isolation was made because the procedure was aimed primarily at the isolation of other compounds. In an earlier report from this laboratory (1) it was noted that material isolated from phenylketonuric urine did not have the physical properties recorded for *o*-HPAA. Previous workers (9, 12) were able to recrystallize *o*-HPAA from chloroform; we have not been able to use chloroform as a solvent for this purpose. It is possible that the chloroform used some time ago may have been impure and was different in solvent action from reagent grade chloroform now in use. Also, the first small amount of *o*-HPAA that was obtained in preliminary experiments crystallized in flat plates and melted at 162°, rather than in prisms melting at 145°. Occasionally, synthetic *o*-HPAA likewise has been obtained in this

high melting form. It is interesting in this regard to note that Blum (12) recorded the isolation of *o*-HPAA which had the properties of *o*-HPAA, but not its melting point. He was able to convert his material into lactone and back into *o*-HPAA having the proper melting point. It seems probable that he may have obtained the higher melting form in his experiment.

The most important question posed by the identification of *o*-HPAA as a metabolite in phenylketonuric urine concerns a possible relation between its formation and the development of the mental defect. The data of Table I indicate that the production of *o*-HPAA is in some manner directly

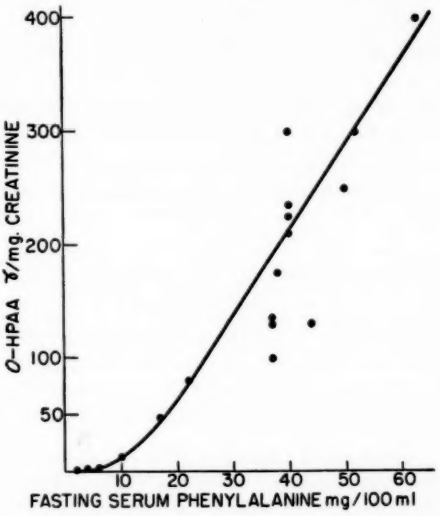


FIG. 2. Excretion of *o*-hydroxyphenylacetic acid in phenylketonuria

related to blood levels of phenylalanine in the patients. This is shown more clearly when the data are presented graphically, as in Fig. 2. Levels for serum phenylalanine and urinary excretion of *o*-HPAA are reported for thirteen patients, in which blood levels of phenylalanine ranged from 22 to 63 mg. per 100 ml. In addition, comparable data are reported which were obtained with two of the patients who were receiving phenylalanine-restricted diets at times when they had different serum levels of phenylalanine. Borek *et al.* (13) have shown that there is no significant correlation between levels of serum phenylalanine and the degree of mental defect in patients with phenylketonuria; hence it seems reasonable to conclude that the production of *o*-HPAA cannot be related in any direct manner to the occurrence of mental defect.

We are indebted to Dr. V. F. Houston, Director of the Utah State Training School for making available to us the facilities of that institution, to Mr. Mark Allen for the psychometric evaluation of the patients, and to to Mrs. Betty Fernelius for her aid in carrying out experiments with the patients at the Training School. We wish to thank Patricia Wall for the analytical work reported here.

SUMMARY

1. *o*-Hydroxyphenylacetic acid has been identified as a constituent of the urine of patients with phenylketonuria. The amount excreted ranges from 100 to 400 mg. per gm. of creatinine. Normal individuals also excrete *o*-hydroxyphenylacetic acid, but at the level of less than 1 mg. per gm. of creatinine.

2. The amount of *o*-hydroxyphenylacetic acid excreted by phenylketonurics is directly related to the phenylalanine levels in the blood. It is probable that the excretion of increased amounts of this metabolite is not related in any direct manner to the occurrence of the mental defect.

BIBLIOGRAPHY

1. Armstrong, M. D., and Robinson, K. S., *Federation Proc.*, **13,** 175 (1954).
2. Jervis, G. A., *Proc. Soc. Exp. Biol. and Med.*, **82,** 514 (1953).
3. Jervis, G. A., *Proc. Soc. Exp. Biol. and Med.*, **75,** 83 (1950).
4. Woolf, L. I., and Vulliamy, D. G., *Arch. Dis. Childhood*, **26,** 487 (1951).
5. Armstrong, M. D., and Tyler, F. H., *J. Clin. Invest.*, in press.
6. Bickel, H., Gerrard, J., and Hickmans, E. M., *Acta paediat.*, **43,** 64 (1954).
7. Armstrong, M. D., and Robinson, K. S., *Arch. Biochem. and Biophys.*, **52,** 287 (1954).
8. Boscott, R. J., and Bickel, H., *Scand. J. Clin. and Lab. Invest.*, **5,** 380 (1953).
9. Czaplicki, S., von Kostanecki, S., and Lampe, V., *Ber. chem. Ges.*, **42,** 827 (1909).
10. Levine, J., Eble, T. E., and Fischbach, H., *J. Am. Chem. Soc.*, **70,** 1930 (1948).
11. Mori, Y., *Z. physiol. Chem.*, **122,** 186 (1922).
12. Blum, L., *Arch. exp. Path. u. Pharmakol.*, **59,** 269 (1908).
13. Borek, E., Brecher, A., Jervis, G. A., and Waelsch, H., *Proc. Soc. Exp. Biol. and Med.*, **75,** 86 (1950).

STUDIES ON PHENYLKETONURIA

III. THE METABOLISM OF *o*-TYROSINE*

By MARVIN D. ARMSTRONG and KENNETH N. F. SHAW

(From the Laboratory for the Study of Hereditary and Metabolic Disorders and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City, and the Utah State Training School, American Fork, Utah)

(Received for publication, October 26, 1954)

The preceding paper from this laboratory reported the isolation and identification of *o*-hydroxyphenylacetic acid (*o*-HPAA) as a constituent of the urine of individuals affected with phenylketonuria (phenylpyruvic oligophrenia) (2). The amount of *o*-HPAA excreted was found to vary directly with the serum phenylalanine levels of the patients. Since it had already been shown by Borek *et al.* (3) that there is no correlation between blood levels of phenylalanine and the severity of the mental defect in phenylketonuria, it appears probable that there is no direct connection between the production of *o*-HPAA and the etiology of the mental defect.

In a preliminary report of the occurrence of *o*-HPAA in phenylketonuric urine, Boscott and Bickel (4) suggested that *o*-HPAA might be present in increased amounts because of a greater production of *o*-tyrosine and also because of an impaired ability in phenylketonuria to oxidize further the *o*-hydroxyphenyl compounds to homogentisic acid. Many years ago, however, Blum (5) reported that administration of *o*-tyrosine leads to the excretion of *o*-HPAA in alkaptonuria, as well as by normal individuals, and that both *o*-tyrosine and *o*-HPAA are metabolized to some extent by both normals and alkaptonurics, and that neither compound gives rise to an increase in homogentisic acid excretion in the alkaptonuric. In addition, Flatow (6) reported that both *o*-tyrosine and *o*-hydroxyphenylpyruvic acid are in part completely metabolized and, in part, excreted as *o*-HPAA by dogs, and Cerecedo and Sherwin (7) found that *o*-HPAA is metabolized to some extent by man, dogs, and rabbits.

This paper reports a study of *o*-tyrosine metabolism in normal individuals and in phenylketonuric patients. The use of paper chromatographic techniques has permitted an extension to near physiological ranges of the older work in which isolation techniques required the use of larger amounts

* This work was supported by research grants from the National Institutes of Health, United States Public Health Service. A preliminary report was presented at the meetings of the American Society of Biological Chemists, Atlantic City, April, 1954 (1).

of compounds. It has been found that there is little, if any, impairment of the ability to handle *o*-hydroxyphenyl compounds in phenylketonuria and that the increased excretion of *o*-HPAA must occur because of a greater production. A hypothesis is advanced to explain the manner in which *o*-tyrosine and, consequently, *o*-HPAA are produced in phenylketonuria, as well as by normal individuals, and the metabolism of *o*-tyrosine is discussed.

EXPERIMENTAL

o-Hydroxyphenylacetic acid (8, 9), DL-*o*-tyrosine (5, 10, 11), and sodium *o*-hydroxyphenyllactate (10, 11) were prepared by modifications of methods described in the literature; the properties of the synthetic materials compared favorably with those reported earlier. Details of the procedures will be reported elsewhere.

Metabolism of o-Hydroxyphenylacetic Acid—Varying amounts of *o*-HPAA were administered orally to normal and to phenylketonuric individuals. Amounts up to 500 mg. were given in one portion; larger amounts, in 500 mg. increments at hourly intervals until the total amount had been ingested. Urine was collected for the following 24 hours, and the amount of *o*-HPAA excreted was estimated chromatographically by the procedure described earlier (2); the results are presented in Table I. Approximately 70 per cent of the extra *o*-HPAA which was excreted appeared in the urine during the first 6 hours after administration. Urine was acidified to pH 1 and autoclaved for 2 hours at 120° before extraction; in addition, extracted urine was subjected to autoclaving and then reextracted. No indication was obtained with these procedures of the presence of any bound *o*-HPAA. The neutral extracts were tested for the presence of the lactone of *o*-HPAA (coumaran-2-one) by prolonged further extraction with 1 N NaOH; no additional *o*-HPAA was found with this treatment.

Metabolism of o-Tyrosine—Varying amounts of DL-*o*-tyrosine were administered to normal and to phenylketonuric individuals, and their urines were examined for *o*-HPAA as described above. The results are presented in Table I. The extracted urine was examined for unmetabolized *o*-tyrosine both directly and by adsorption of the aromatic compounds on activated charcoal, followed by elution; under these conditions, small amounts of *o*-tyrosine which had been added to urine could be detected readily. No *o*-tyrosine was found normally in phenylketonuric urine or after the administration of *o*-tyrosine either to normals or phenylketonurics.

Because of the occurrence of phenyllactic acid, indolelactic acid (12), and *p*-hydroxyphenyllactic acid (1, 4) in phenylketonuric urine, it might have been expected that *o*-hydroxyphenyllactic acid (*o*-HPLA) also would be present. No *o*-HPLA could be detected in the urine of patients either after concentration of extracts of their urine or upon examination of the

fractions obtained by counter-current distribution. If *o*-HPLA is present, the quantity is extremely small and must amount to less than 0.1 per cent of the amount of *o*-HPAA. In addition, *o*-HPLA could not be detected

TABLE I

Metabolism of o-Hydroxyphenylacetic Acid and DL-*o-Tyrosine*

	o-Hydroxyphenylacetic acid			*o*-Tyrosine		
	Ingested	Excreted	Excretion of ingested *o*-HPAA	Ingested (as *o*-HPAA)	Excreted (as *o*-HPAA)	Excretion of ingested *o*-tyrosine
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
Normal	0	1		0	1	
	10	10.3	93	10	7.5	65
	50	48	94	50	34	66
	100	84	83	100	58	57
	500	397	80	500	235	47
	1000	700	70	1000	340	34
Phenylketonuric A	0	180		0	175	
	500	570	80	1000	435	26
" B	0	115		0	135	
	1000	955	84	500	200	40
" C	0	40		0	40	
	250	280	95	250	147	43
" D	0	61		0	61	
	500	445	77	500	230	34

TABLE II

*Metabolism of o-Hydroxyphenyl-*DL-*Lactic Acid in Normal Human*

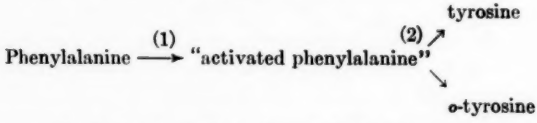
Sodium *o*-hydroxyphenyllactate ingested	Excreted		*o*-HPLA not metabolized	Metabolized *o*-HPLA excreted as *o*-HPAA
	o-HPLA	*o*-HPAA		
mg.	*mg.*	*mg.*	*per cent*	*per cent*
50	17	22	38	92
100	35	35	39	76
500	200	160	45	78
1000	500	225	56	69

in the urine of normal or phenylketonuric individuals who had ingested *o*-tyrosine. *o*-HPLA was administered to normal individuals and extracts of their urine were examined for the presence of *o*-HPAA and of unchanged *o*-HPLA (R_F = 0.52 with isopropyl alcohol-aqueous ammonia-H_2O = 8:1:1; orange dye with diazotized sulfanilic acid). The results of these experiments appear in Table II.

DISCUSSION

The first point to consider concerning the origin of the *o*-HPAA that is excreted in phenylketonuria is whether the greatly increased excretion of *o*-HPAA occurs because of an increased production of the metabolite, because of a decreased ability to metabolize it, or a combination of both factors. The results (Table I) indicate that there is little difference between normals and phenylketonurics in the manner in which they metabolize *o*-HPAA. Further, there is little difference in the manner in which DL-*o*-tyrosine is handled in each (Table I). These data indicate that the excretion of greatly increased quantities of *o*-HPAA in phenylketonuria occurs because of the formation of much larger amounts rather than because of an impaired ability to oxidize it, and might have been anticipated from the earlier work.

In seeking the origin of *o*-HPAA in human urine, it is reasonable to assume that *o*-tyrosine or some closely related substance is formed and serves as a precursor. It is, then, necessary to explain the manner of formation of small amounts of *o*-tyrosine in normal individuals and to account for the increased production in phenylketonuria. From the data presented previously (2), it can be estimated that an average adult phenylketonuric excretes approximately 200 mg. of *o*-HPAA daily; this value corresponds to the formation of about 480 mg. of *o*-tyrosine, since only 50 per cent of administered *o*-tyrosine appears in the urine as *o*-HPAA (Table I). It can be estimated that the phenylketonuric patients reported here consumed about 70 gm. of protein daily, since they were all in a good state of nutrition; this amount of protein would contain about 3.5 gm. of phenylalanine. Thus, about 12 per cent of the ingested phenylalanine is converted to *o*-tyrosine. Udenfriend and Bessman (13), using C^{14}-labeled phenylalanine, have found that tyrosine is formed from phenylalanine by phenylketonuric patients, but at a rate only 7 to 10 per cent of that of normals. Thus, tyrosine and *o*-tyrosine are formed by the phenylketonuric individual in approximately equal amounts. This ratio is in reasonable agreement with that expected for ortho-para substitution of an alkyl benzene by electrophilic attack. Mitoma and Leeper (14) have reported that the enzyme system in mammalian liver extract which carries out the oxidation of phenylalanine to tyrosine consists of at least two separate enzymes. It may be suggested that the oxidation of phenylalanine in the mammalian liver proceeds in the following manner.



Normally, most of the ingested phenylalanine in excess of the amount

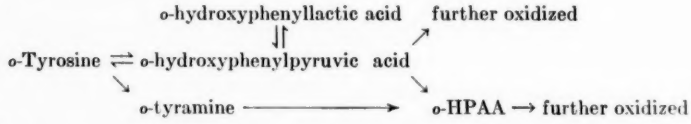
required for new protein synthesis is thought to be converted to tyrosine and then further oxidized. The excretion of approximately 1 mg. of *o*-HPAA by a normal individual would indicate the production of 2 mg. of *o*-tyrosine, corresponding to only 0.05 per cent of the ingested phenylalanine. If the enzyme responsible for carrying out Step 2 is the one which is impaired in phenylketonuria, considerable amounts of "activated phenylalanine" might accumulate and be subjected to non-specific chemical attack by biochemical oxidizing agents of electrophilic nature. This would lead to the formation of tyrosine and *o*-tyrosine in the observed relative amounts.

The pathway for the catabolism of phenylalanine through *o*-tyrosine is obviously of little quantitative significance in the normal individual. In phenylketonuria, however, it becomes important as a means for disposal of some of the accumulated phenylalanine. Older studies failed to account for all of the phenylalanine ingested by phenylketonuric patients; only about 60 per cent could be measured as phenylalanine, phenyllactic acid, and phenylpyruvic acid in the urine (15). Woolf (16) and Stein *et al.* (17) have reported that a considerable amount of phenylacetic acid appears in the urine of phenylketonuric patients as phenylacetylglutamine, but it is possible that phenylacetylglutamine was determined as phenyllactic acid in the earlier work (15). In any case, the findings that as much as 10 per cent of ingested phenylalanine may be oxidized to tyrosine (13) and that at least 10 per cent may form *o*-tyrosine now account for most of the phenylalanine metabolized by these patients.

The final subject for consideration is the pathway by which *o*-hydroxyphenylacetic acid is formed from *o*-tyrosine. Blaschko (18) has reviewed the earlier literature on the suggested pathways for the metabolism of *o*-tyrosine. He found that DL-*o*-tyrosine is a substrate for both D- and L-amino acid oxidases; therefore it seemed possible that *o*-HPAA might be formed from *o*-tyrosine via *o*-hydroxyphenylpyruvic acid as an intermediate. No studies of transamination with *o*-tyrosine have been reported to date. Blaschko found, however, that *o*-tyrosine is also an excellent substrate for a decarboxylase present in guinea pig liver or kidney extracts. Since Randall (19) had reported earlier that *o*-tyramine is a substrate for amine oxidase, Blaschko suggested that *o*-HPAA might be formed by decarboxylation of *o*-tyrosine, followed by the action of amine oxidase on *o*-tyramine.

The results presented here are quite definite in showing that the recovery of *o*-tyrosine as *o*-HPAA is distinctly lower than the recovery of *o*-HPAA itself, in both phenylketonurics and normals. This indicates that some pathway for the metabolism of *o*-tyrosine other than that by way of *o*-HPAA exists. No significant amount of *o*-tyrosine was detected in the urine of persons who had received *o*-tyrosine, and the amounts used

in these experiments were so small as to preclude the loss of a significant amount of material because of lack of intestinal absorption. There was no excretion of *o*-HPLA after the administration of *o*-tyrosine. No indication of the excretion of *o*-hydroxyphenylpyruvic acid was obtained, urinary *o*-HPAA did not occur in a bound form which would have escaped extraction, nor was any lactone detected. Thus, it is probable that a considerable portion of ingested *o*-tyrosine is metabolized completely. The accompanying scheme could represent the pathway by which *o*-tyrosine is metabolized.



It is not possible at present to decide which pathway is more important for the formation of *o*-HPAA. The experiments with *o*-HPLA were carried out in an attempt to gain some information in this regard. The data show that recovery of the portion of *o*-HPLA which was metabolized to *o*-HPAA resembles that found with *o*-HPAA itself rather than that with *o*-tyrosine. The facts that racemic forms of *o*-tyrosine and of *o*-HPLA were used and that the site at which the enzymatic reactions take place (liver, kidney?) is not known make it impossible to draw any definite conclusions as to pathways from this experiment. Further work at the enzyme level will be necessary to decide between the alternative pathways.

It is possible that the aromatic ring might be oxidized at the pyruvic acid stage more readily than at the acetic acid stage. In this regard, it is interesting to note that little work has been done on the corresponding stage of the oxidation of tyrosine. Some earlier workers felt that homogentisic acid might not be a normal intermediate on the pathway for tyrosine oxidation (20), even though it is observed in the urine of animals under some unphysiological conditions and can be oxidized by animals and by extracts of animal tissues. A comparative study of the oxidation of *o*-hydroxyphenylpyruvic acid and 2,5-dihydroxyphenylpyruvic acid and of the corresponding acetic acids might give some information on this subject. The data in Table I indicate that an increasing proportion of the *o*-hydroxy compounds is metabolized as increasing amounts are administered. This is the reverse of the situation observed with many compounds, in which an increase in the amount given leads to a decreased amount metabolized. These data are in accord with the results which would be expected if *o*-HPAA and *o*-HPLA were metabolized by the same enzyme system normally concerned with the oxidation of tyrosine and were competing with the normal metabolites for the action of this system. It might be expected that an impaired metabolism of *o*-HPAA and *o*-tyro-

sine occurs in alkaptonuria. Unfortunately, Blum did not study the recovery of *o*-HPAA in his experiments with alkaptonurics, but established only that *o*-HPAA or *o*-tyrosine administration did not lead to an increased excretion of homogentisic acid.

We are indebted to Dr. V. F. Houston, Director of the Utah State Training School, for making available to us the facilities of that institution, and to Mrs. Betty Fernelius for her aid in carrying out metabolic experiments with the patients at the Training School. We wish to thank Patricia Wall for the analytical work reported here.

SUMMARY

1. The increased amount of *o*-hydroxyphenylacetic acid in phenylketonuric urine has been shown to occur as the result of an increased production rather than an impaired mechanism for further oxidation.

2. The metabolism of DL-*o*-tyrosine has been studied in both normal and phenylketonuric individuals. There appears to be no abnormality in the manner in which *o*-tyrosine is metabolized in phenylketonuria.

3. The possible origin and the metabolism of *o*-tyrosine, the probable precursor of *o*-hydroxyphenylacetic acid, are discussed.

BIBLIOGRAPHY

1. Armstrong, M. D., and Robinson, K. S., *Federation Proc.*, **13,** 175 (1954).
2. Armstrong, M. D., Shaw, K. N. F., and Robinson, K. S., *J. Biol. Chem.*, **213,** 797 (1955).
3. Borek, E., Brecher, A., Jervis, G. A., and Waelsch, H., *Proc. Soc. Exp. Biol. and Med.*, **75,** 86 (1950).
4. Boscott, R. J., and Bickel, H., *Scand. J. Clin. and Lab. Invest.*, **5,** 380 (1953).
5. Blum, L., *Arch. exp. Path. u. Pharmakol.*, **59,** 269 (1908).
6. Flatow, L., *Z. physiol. Chem.*, **64,** 367 (1910).
7. Cerecedo, L. R., and Sherwin, C. P., *J. Biol. Chem.*, **58,** 215 (1923–24).
8. Czaplicki, S., von Kostanecki, S., and Lampe, V., *Ber. chem. Ges.*, **42,** 827 (1909).
9. Levine, J., Eble, T. E., and Fischbach, H., *J. Am. Chem. Soc.*, **70,** 1930 (1948).
10. Plöchl, J., and Wolfram, L., *Ber. chem. Ges.*, **18,** 1183 (1885).
11. Dakin, H. D., *J. Biol. Chem.*, **82,** 439 (1929).
12. Armstrong, M. D., and Robinson, K. S., *Arch. Biochem. and Biophys.*, **52,** 287 (1954).
13. Udenfriend, S., and Bessman, S. P., *J. Biol. Chem.*, **203,** 961 (1953).
14. Mitoma, C., and Leeper, L. C., *Federation Proc.* **13,** 266 (1954).
15. Jervis, G. A., *Proc. Soc. Exp. Biol. and Med.*, **75,** 83 (1950).
16. Woolf, L. I., *Biochem. J.*, **49,** p. ix (1951).
17. Stein, W. H., Paladini, A. C., Hirs, C. H. W., and Moore, S., *J. Am. Chem. Soc.*, **76,** 2848 (1954).
18. Blaschko, H., *Biochem. J.*, **44,** 268 (1949).
19. Randall, L. O., *J. Pharmacol. and Exp. Therap.*, **88,** 216 (1946).
20. Dakin, H. D., Oxidations and reductions in the animal body, Monographs on biochemistry, London, 85 (1922).

XUM

ISOLATION AND PROPERTIES OF YEAST GLUTATHIONE SYNTHETASE*

By JOHN E. SNOKE†

(*From the Department of Biochemistry, University of Chicago, Chicago, Illinois*)

(Received for publication, October 9, 1954)

The biosynthesis of GSH[1] from its constituent amino acids, as reported in a previous communication from this laboratory (3), involves the intermediary formation of γ-glutamylcysteine. The formation of this dipeptide and the subsequent condensation of γ-glutamylcysteine with glycine are catalyzed by separate enzymes in reactions which are dependent upon ATP (3). The enzyme which catalyzes the synthesis of GSH from glutamylcysteine and glycine has been obtained in a partially purified state from extracts of acetone-dried pigeon liver and some of its properties have been investigated (4). An enzyme which catalyzes the same reaction has now been obtained in a high state of purity from brewers' yeast. In the present communication, the isolation of the enzyme, as well as some of its properties, is described. The name glutathione synthetase is proposed for the enzyme.

EXPERIMENTAL

Materials—C^{14}-Glycine, L-γ-glutamyl-L-cysteine, aminomethanesulfonic acid, sarcosine, and the lyophilized rabbit muscle extract were the same preparations that had been used previously (3, 4). GSH was a product of the Schwarz Laboratories, and ATP was a commercial preparation which was purified on a Dowex 1 column according to the procedure of Cohn and Carter (5). Nucleic acid, protamine sulfate, and 3-PGA were commercial preparations. UTP and ITP were kindly supplied by Dr. Eugene Kennedy.

Methods—The enzymatic synthesis of GSH from glutamylcysteine and glycine was carried out in stoppered test-tubes at 37°. The composition

* Supported by grants-in-aid from the Division of Research Grants and Fellowships of the United States Public Health Service and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. Some of the data presented in this paper have appeared in preliminary publications (1, 2).

† Present address, Department of Physiological Chemistry, University of California Medical Center, Los Angeles, California.

[1] The following abbreviations are used: GSH, glutathione; ATP, ADP, AMP, UTP, ITP, adenosinetri-, adenosinedi-, adenosinemono-, uridinetri-, inosinetriphosphate; Tris, tris(hydroxymethyl)aminomethane; 3-PGA, 3-phosphoglyceric acid; Pi, orthophosphate.

XUM

of the incubation mixtures is described in Tables I to VI and Figs. 1 to 5. GSH synthesis was followed either by isotopic assay (6) or by measuring the release of Pi from ATP. Inorganic phosphate was determined by the method of Gomori (7) and hydroxamic acid formation was measured according to the procedure of Lipmann and Tuttle (8). Protein concentrations were determined by the method of Bücher (9). The energy requirements of the system were met by adding either ATP alone or by the generation of ATP with the aid of 3-PGA in the presence of a lyophilized rabbit muscle extract which contained the appropriate glycolytic enzymes (10).

Results

Isolation of Enzyme

Autolysis and First $(NH_4)_2SO_4$ *Fractionation*—Brewers' yeast[2] was air-dried at room temperature and pulverized in a hammer mill. The yeast was autolyzed by heating for 4 hours at 37° with 3 volumes of distilled water. An additional 3 volumes of water were then added and the mixture was centrifuged. To each liter of supernatant fluid were added 333 gm. of solid ammonium sulfate and the mixture was allowed to stand overnight. The precipitate was collected by centrifugation and dissolved in water to one-fourth the original volume of the autolysate.

Second $(NH_4)_2SO_4$ *Fractionation*—The concentration of ammonium sulfate in the above solution was estimated by nesslerization and sufficient solid ammonium sulfate was added so that the concentration of salt was 260 gm. per liter. The pH was adjusted to 8.0 by the addition of 3.0 N ammonium hydroxide and the mixture was allowed to stand overnight. The precipitate was removed by centrifugation and 82 gm. of solid ammonium sulfate were added per liter of supernatant fluid. After stirring for 1 hour, the precipitate was collected by centrifugation and dissolved in a minimum of water and the solution dialyzed overnight against running tap water.

Acid Precipitation and Partial Heat Denaturation—The dialyzed solution was adjusted to pH 4.5 by the addition of 0.2 N sulfuric acid and brought to 42° by rapid stirring in a water bath kept at 60°. The mixture was kept at 42° for 30 minutes and then cooled. The precipitate was removed by centrifugation.

Third and Fourth $(NH_4)_2SO_4$ *Fractionation*—To each liter of supernatant fluid were added 280 gm. of solid ammonium sulfate. The mixture was stirred for 1 hour, and the precipitate was collected by centrifugation and then dissolved in water. To each liter of solution were added 450 ml. of

[2] We are indebted to Mr. Gelman and Dr. Kaplan of Vico Products Company, Chicago, Illinois, for generously supplying us with the yeast.

a saturated ammonium sulfate solution and the pH was adjusted to 4.5 by the addition of 0.2 N H_2SO_4. After 1 hour of stirring, the precipitate

TABLE I

Purification of Yeast Enzyme

Step	Volume	Protein	Total activity*	Specific activity†
	ml.	*mg. per ml.*		
Autolysate	150,000	42.5	2,180,000	0.34
1st $(NH_4)_2SO_4$ fraction	35,800	47.5	1,620,000	0.95
2nd " "	9,600	58.0	1,350,000	2.43
Acid ppt. and heat denaturation	9,700	10.0	950,000	9.80
3rd $(NH_4)_2SO_4$ fraction	955	22.0	614,000	35.7
4th " "	430	9.0	372,000	96.4
Nucleic acid fraction	137	5.20	298,000	419
Alcohol fraction	16.5	6.10	194,000	1930

The starting material was 33.5 kilos of dried brewers' yeast. The activity of the various fractions was determined at pH 8.3 and 37° by incubating the enzyme for 30 minutes in the presence of 0.1 M Tris buffer, 0.1 M KCl, 0.01 M $MgSO_4$, 0.015 M KCN, 0.002 M glutamylcysteine, 0.015 M C^{14}-glycine, 0.003 M ATP, 0.005 M 3-PGA, 1.0 mg. of serum albumin, and 1.0 mg. of rabbit muscle preparation. The total volume of the reaction mixture was 1.0 ml. The amount of enzyme was chosen to provide synthesis of GSH at a rate linear with respect to time and proportional to the enzyme concentration.

* Expressed as micromoles of GSH synthesized per 30 minutes.

† Expressed as micromoles of GSH synthesized per 30 minutes per mg. of protein.

TABLE II

Balance Study of GSH Synthesis

	GSH synthesized	Pi	AMP	ADP	ATP
Initial	0	0	0.04	0.45	1.18
Final	0.48	0.49	0.04	0.89	0.71
Δ	+0.48	+0.49	0	+0.44	−0.47

The reaction mixture, 20.0 ml. in volume, contained 0.01 M Tris, 0.005 M KCN, 0.004 M $MgSO_4$, 0.005 M C^{14}-glycine, 0.002 M glutamylcysteine, 0.4 per cent serum albumin, and 0.0075 mg. of enzyme protein per ml. of reaction mixture. The enzyme preparation was approximately 1500-fold purified. The incubation was for 60 minutes at 37°, pH 8.3. The nucleotides were determined by Siekevitz and Potter's modification (11) of the Dowex 1 technique of Cohn and Carter (5). The results are expressed in micromoles per ml. of reaction mixture.

was removed by centrifugation and an additional 250 ml. of saturated ammonium sulfate were added. The mixture was stirred again for 1 hour

and centrifuged. The precipitate was dissolved in H_2O, and the solution was dialyzed overnight against running tap water.

Nucleic Acid Fractionation—The above dialyzed solution was adjusted to contain 5.0 mg. of protein per ml. and 5.0 ml. of nucleic acid solution (2.5 gm. of nucleic acid and 3.75 ml. of 1 N sodium hydroxide per 50 ml.) were added for every 100 ml. of protein solution. The pH was adjusted

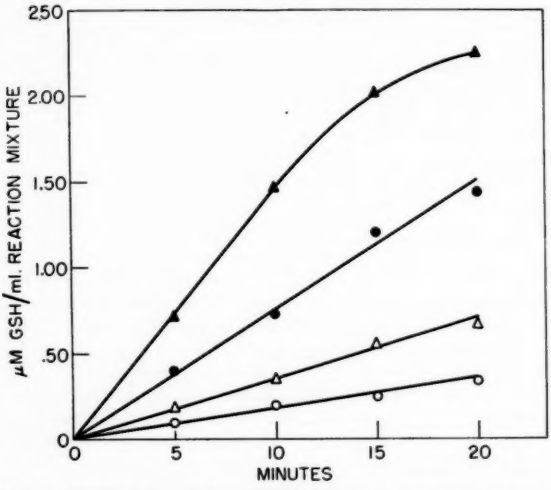


FIG. 1. Rate of GSH synthesis from glutamylcysteine at various enzyme concentrations. The reaction mixtures, 5.0 ml., contained 0.1 M Tris, 0.1 M KCl, 0.01 M $MgSO_4$, 0.015 M KCN, 0.015 M C^{14}-glycine, 0.0025 M glutamylcysteine, 0.003 M ATP, 0.005 M 3-PGA, 1.0 mg. of serum albumin per ml., and 1.0 mg. of rabbit muscle extract per ml. of reaction mixture. The amount of enzyme in mg. of protein per ml. is given by ▲ 21.6×10^{-4}, ● 10.8×10^{-4}, △ 5.4×10^{-4}, and ○ 2.7×10^{-4}. At the time intervals indicated 1.0 ml. aliquots were withdrawn and the amount of GSH synthesized was determined.

to 5.35 by the dropwise addition of 0.005 N sulfuric acid and the mixture stirred for 30 minutes. The precipitate was removed by centrifugation and the supernatant liquid adjusted to pH 4.90. After stirring for 30 minutes the precipitate was collected by centrifugation and dissolved by suspension in water and the pH was carefully adjusted to 7.0 by the dropwise addition of 0.03 N sodium hydroxide. The nucleic acid was removed by the addition of protamine sulfate (20 mg. per ml.). The addition of protamine sulfate was continued until no further precipitation occurred.[3]

[3] The results appearing in preliminary publications (1, 2) were obtained with an enzyme preparation which was carried through the nucleic acid fractionation and

Alcohol Fractionation—To the supernatant fluid obtained after the protamine treatment 2 volumes of saturated ammonium sulfate were added. The precipitate was collected by centrifugation and dissolved in approximately 10 ml. of water, and the solution was dialyzed against distilled water until salt-free. The solution was adjusted to contain 6 mg. of protein per ml., 0.02 M phosphate buffer (pH 6.7), and 0.06 M sodium chloride, and 18 ml. of absolute ethanol were added dropwise with stirring

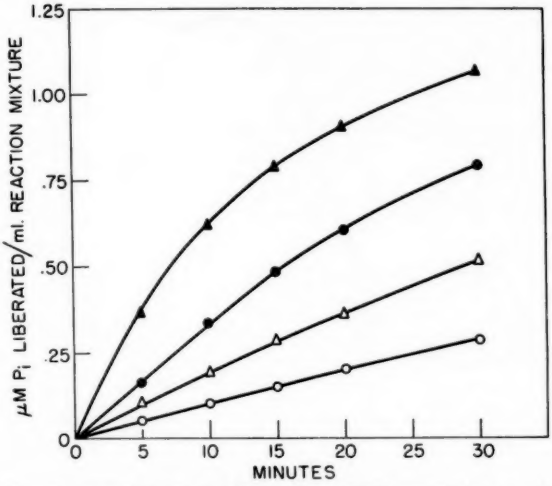


FIG. 2. Phosphate release from ATP during GSH synthesis at various enzyme concentrations. The compositions of the reaction mixtures were identical to those given in Fig. 1, except that the rabbit muscle extract and 3-PGA were omitted. The amount of enzyme in mg. of protein per ml. is given by ▲ 21.6×10^{-4}, ● 10.8×10^{-4}, △ 5.4×10^{-4}, and ○ 2.7×10^{-4}. At the time intervals indicated 1.0 ml. aliquots were withdrawn and the amount of Pi liberated was determined.

for every 100 ml. of solution. The temperature of the mixture was lowered from 0° to −4° during the ethanol addition. After stirring for 15 minutes, the mixture was centrifuged at −4°. To the supernatant fluid were added 28 ml. of ethanol for every 100 ml. of original solution, and the temperature was lowered to −10° during the addition of the ethanol. The mixture was stirred for 15 minutes and centrifuged at −10°. The ethanol was removed from the precipitate by lyophilization, and the precipitate was dissolved in 0.25 M ammonium sulfate. A summary of the fractionation procedure is given in Table I.

which was approximately 1500-fold purified. As a result of miscalculation, the enzyme protein concentrations given in these publications are twice their actual values.

Unless noted otherwise all fractionation procedures were carried out at 0°. The first two steps of the fractionation were carried out in ten separate batches and in five separate batches during the next three steps. The enzyme is completely stable when stored either in the frozen state at −20° or stored in the cold after lyophilization. In the purified state, the enzyme is slowly inactivated during the conditions used for assay. This

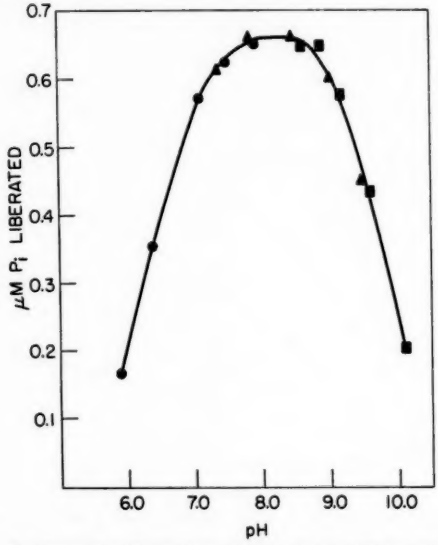


FIG. 3. Effect of pH on the liberation of Pi due to GSH synthesis. The experimental conditions were identical to those given in Fig. 2, except that the buffer was varied. The buffers used were ● 0.1 M imidazole, ▲ 0.10 M Tris, and ■ 0.03 M glycine. The reaction mixtures, 1.0 ml., were incubated for 30 minutes at 37°. The amount of enzyme present in the above systems was 9.7×10^{-4} mg. of protein per ml.

inactivation is prevented by performing the incubations in 0.1 per cent serum albumin.

The most highly purified fraction has been examined in the ultracentrifuge and found to sediment as two components, which contain approximately 85 and 15 per cent of the total protein. None of the numerous attempts to crystallize the enzyme has met with success.

Stoichiometry of Reaction—With the purified yeast enzyme a chemical balance study of GSH synthesis has been made. The data shown in Table II demonstrate that the condensation of glutamylcysteine and glycine is accompanied by the splitting of ATP to yield ADP and Pi in amounts

equivalent to the GSH formed in accordance with the equation

L-γ-Glutamyl-L-cysteine + glycine + ATP → GSH + ADP + Pi

Effect of Enzyme Concentrations—In Fig. 1 are shown the rates of GSH synthesis obtained over an 8-fold range of enzyme concentration. In

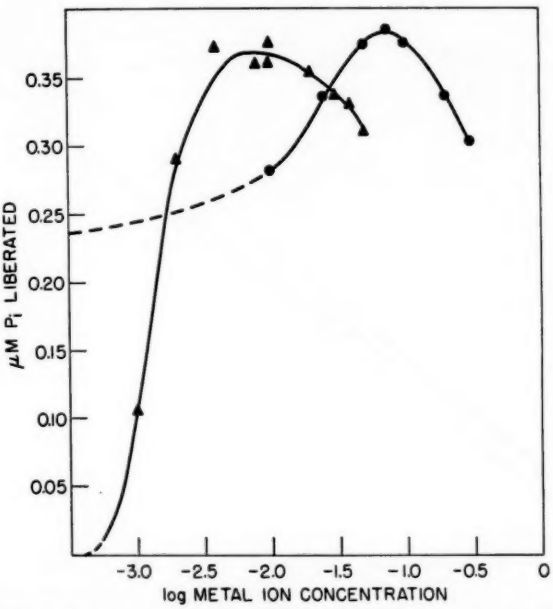


FIG. 4. Effect of magnesium and potassium ions on Pi release due to GSH synthesis. The experimental conditions were identical to those given in Fig. 2 except for the variation in the metal ion concentrations. In the experiment in which the potassium concentration was varied (●), the magnesium concentration was 0.01 M. In the experiment in which the magnesium concentration was varied (▲), the potassium concentration was 0.10 M. The reaction mixture, 1.0 ml., was incubated for 30 minutes and contained 3.4×10^{-4} mg. of enzyme protein.

these experiments ATP was continuously regenerated and glycine was not limiting. Under these conditions the rate of synthesis is proportional to enzyme concentration and follows zero order kinetics until the concentration of glutamylcysteine becomes limiting.

The rates of GSH formation in the absence of the ATP-generating system as measured by Pi release are shown in Fig. 2. Under these conditions the rate of GSH synthesis gradually declines because of inhibition by ADP. These results are similar to those obtained previously with the

pigeon liver enzyme (4, 12). The release of Pi from ATP can be used as an accurate assay of GSH synthesis, provided that the ratio of ADP to ATP remains low under the conditions used.

Effect of pH—The effect of pH on the synthesis of GSH from glutamylcysteine and glycine as measured by the liberation of Pi from ATP is

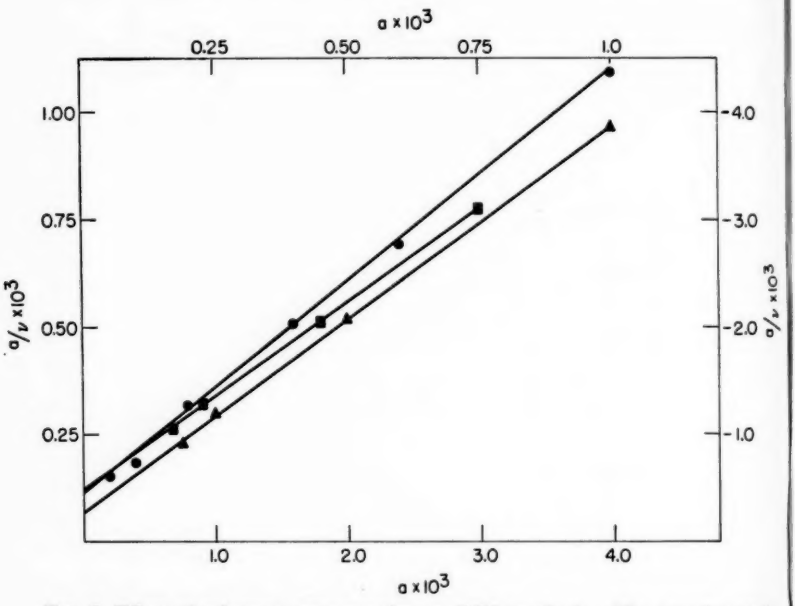


FIG. 5. Effect of substrate concentration on GSH synthesis. The experimental conditions were identical to those given in Fig. 1, except that the concentrations of glycine ■, glutamylcysteine ▲, and ATP ● were varied. The rate of synthesis, ν, is expressed in micromoles of GSH per 60 minutes and the concentration of the substrates, a, is in moles per liter. The experiments in which the concentration of ATP was varied should be referred to the ordinate on the left and to the upper abscissa. The amount of enzyme in each system was 3.2×10^{-4} mg. of protein per ml.

shown in Fig. 3. The enzyme is active over a fairly broad pH range with an optimum at 8.3.

Effect of Cations—The effect of magnesium and potassium ions on GSH formation is presented in Fig. 4. There is an absolute requirement for magnesium ions, the concentration being optimal at 0.005 M. Potassium ions produce a marked stimulation of GSH synthesis, an effect which is optimal at 0.08 M. Magnesium and potassium ions cannot be replaced by other divalent and monovalent cations.

Effect of Substrate Concentrations—The initial rate of GSH synthesis was determined at various concentrations of ATP, glutamylcysteine, and glycine. The data were plotted according to one of the Lineweaver-Burk equations (13) as shown in Fig. 5. The K_m values calculated from the data in Fig. 5 are 1.1×10^{-4} M, 2.9×10^{-4} M, and 5.8×10^{-4} M for ATP, glutamylcysteine, and glycine, respectively.

Specificity—The liberation of Pi from ATP which accompanies GSH synthesis has been used as a means of determining whether glutamylcysteine can condense with compounds other than glycine. In Table III

TABLE III

Replacement of Glycine by Various Acceptors

Acceptor	Concentration	Phosphate liberated
	M	μmole
Glycine	0.02	0.58
Hydroxylamine	0.04	0.07
"	0.10	0.15
"	0.40	0.25
Aminomethanesulfonic acid	0.02	0.19
" "	0.10	0.36
L-Alanine	0.10	0.04
Sarcosine	0.10	0
Glycolic acid	0.10	0
Methylamine	0.10	0
Semicarbazide	0.10	0

The experimental conditions were the same as those given in Fig. 2, except that glycine was replaced by the various compounds shown. The reaction mixtures, 1.0 ml. in volume, were incubated for 30 minutes. The amount of enzyme in the above system was 8×10^{-4} mg. of protein per ml.

are given the amounts of Pi which are liberated upon addition of various compounds. When tested at 0.02 M, the only compound apart from glycine that caused the release of significant amounts of Pi from ATP was aminomethanesulfonic acid. The yeast enzyme differs in this regard from the previously studied pigeon liver enzyme which failed to catalyze the release of Pi when glycine was replaced by the sulfonic acid analogue (4). Pi release does occur also with high concentrations of hydroxylamine. This release of Pi is a quantitative measure of hydroxamic acid formation as demonstrated by the data in Table IV.

Table V shows the results of attempts to replace ATP by other nucleotides. Although it would appear that a small amount of GSH synthesis occurred in the presence of UTP, this result could be due to contamination of the UTP preparation by as little as 10 per cent ATP. The lack of

TABLE IV

Formation of Hydroxamic Acid from Glutamylcysteine, Hydroxylamine, and ATP

Time	Hydroxamic acid	Phosphate liberated
min.	*μmole per ml.*	*μmole per ml.*
20	0.27	0.29
40	0.47	0.47
60	0.62	0.63

The experimental conditions were the same as those given in Fig. 2, except that glycine was replaced by 0.40 M hydroxylamine and the total volume of the reaction mixture was 10 ml. At the time intervals indicated, 1.0 ml. and 2.0 ml. aliquots were withdrawn for Pi and hydroxamic acid determinations, respectively. Succinic anhydride was used as a standard in the hydroxamic acid assay. The amount of enzyme present was 1.1×10^{-3} mg. of protein per ml. of reaction mixture.

TABLE V

Replacement of ATP by Other Nucleotides

Nucleotide	GSH synthesized
	μmole
ATP	0.52
ADP	0
AMP	0
UTP	0.10
ITP	0.02
UTP + ADP	0.04
ITP + "	0.01

The experimental conditions were the same as those given in Fig. 2, except that C^{14}-glycine was used and the ATP was replaced by the nucleotides shown. The concentration of nucleotide was 0.003 M, except when ITP and UTP were used in combination with ADP. In these experiments the concentration of each nucleotide was 0.0015 M. The reaction mixtures, 1.0 ml. in volume, were incubated for 60 minutes and contained 1.6×10^{-4} mg. of protein.

TABLE VI

Sulfhydryl-Activated ATPase Activity

Additions	Phosphate liberated
	μmole
None	0
0.016 M glutamylcysteine	0.76
0.016 " GSH	0.16
0.016 " cysteine	0.44
0.010 " glycine	0

The reaction mixture, 1.0 ml. in volume, contained 0.1 M Tris buffer, 0.1 M KCl, 0.015 M KCN, 0.003 M ATP, 1.0 mg. of serum albumin, and 0.276 mg. of enzyme. The incubation was carried out at 37°, pH 8.3, for 60 minutes.

GSH synthesis in the presence of UTP and ITP in combination with ADP demonstrates the absence of an enzyme which displays the activity of nucleoside diphosphokinase as described by Berg and Joklik (14).

ATPase Activity—The release of Pi from ATP provides an accurate index of GSH synthesis, since, under conditions used for the assay, the enzyme causes the liberation of detectable amounts of Pi only when the system is complete, *i.e.* when both glutamylcysteine and glycine are present. In the presence of large amounts of the enzyme, however, a definite release of Pi from ATP occurs, providing a sulfhydryl compound is present. This "ATPase" action is proportional both to enzyme concentration and time. In Table VI are shown the amounts of Pi released under various conditions. Although glutamylcysteine was more active than cysteine or GSH in promoting this apparent ATPase action, it is not clear whether this activity is an intrinsic property of glutathione synthetase.

DISCUSSION

The properties of glutathione synthetase isolated from yeast are practically identical with those of the previously studied enzyme from pigeon liver (4). The only difference between the two enzymes that has been observed is that in the yeast system aminomethanesulfonic acid can condense with glutamylcysteine, whereas the pigeon liver enzyme appears to be completely specific for glycine. The fractionation of the yeast enzyme, as that of pigeon liver (4), failed to resolve the enzymic activity into more than one component. In view of these results, and in view of the high degree of purification of the yeast enzyme, it may be concluded that the formation of GSH from glutamylcysteine and glycine is catalyzed by a single enzymic component.

The equivalence between the amounts of tripeptide formed and the liberation of Pi and ADP from ATP provide evidence that the energy required for the synthesis of GSH is obtained through the splitting of the terminal phosphate of ATP. As has been previously concluded from the data obtained with the pigeon liver enzyme (4), the formation of a hydroxamic acid from glutamylcysteine, hydroxylamine, and ATP suggests that an activated glutamylcysteine is formed. The chemical nature of this activated glutamylcysteine and the details of the mechanism by which ATP is utilized have nevertheless remained obscure.

SUMMARY

The enzyme which catalyzes the formation of GSH from glutamylcysteine, glycine, and ATP has been purified approximately 5500-fold from an autolysate of brewers' yeast. The synthesis of GSH has an absolute requirement for magnesium ions and is markedly stimulated by potassium

ions. The effects of enzyme and substrate concentrations, the pH optimum, and partial specificity requirements have been studied. The formation of glutathione is accompanied by the splitting of ATP to ADP and Pi in amounts equivalent to the tripeptide formed.

BIBLIOGRAPHY

1. Snoke, J. E., *J. Am. Chem. Soc.*, **75**, 4872 (1953).
2. Snoke, J. E., and Bloch, K., in Colowick, S. P., Glutathione, New York, 129 (1954).
3. Snoke, J. E., and Bloch, K., *J. Biol. Chem.*, **199**, 407 (1952).
4. Snoke, J. E., Yanari, S., and Bloch, K., *J. Biol. Chem.*, **201**, 573 (1953).
5. Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72**, 4273 (1950).
6. Johnston, R. B., and Bloch, K., *J. Biol. Chem.*, **188**, 221 (1951).
7. Gomori, G., *J. Lab. and Clin. Med.*, **27**, 955 (1942).
8. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).
9. Bücher, T., *Biochim. et biophys. acta*, **1**, 292 (1947).
10. Ratner, S., and Pappas, A., *J. Biol. Chem.*, **179**, 1183 (1949).
11. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **200**, 187 (1953).
12. Yanari, S., Snoke, J. E., and Bloch, K., *J. Biol. Chem.*, **201**, 561 (1953).
13. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
14. Berg, P., and Joklik, W. K., *Nature*, **172**, 1008 (1953).

STUDIES ON THE MECHANISM OF ACTION OF GLUTATHIONE SYNTHETASE*

By JOHN E. SNOKE† and KONRAD BLOCH‡

(From the Department of Biochemistry, University of Chicago, Chicago, Illinois)

(Received for publication, October 9, 1954)

It has been established that the synthesis of GSH[1] from glutamylcysteine and glycine is accompanied by the splitting of ATP to ADP and Pi, but the detailed mechanism of this process has not been elucidated. It is reasonable to assume that the formation of the peptide bond which is catalyzed by glutathione synthetase occurs in several steps. Nevertheless, present evidence indicates that the reaction is catalyzed by a single enzyme (3, 4). Also, it has not been possible so far to accumulate intermediates or to ascertain their chemical nature. It is likely therefore that the intermediates that are formed in the course of the reaction are bound to the enzyme, possibly by covalent linkages. In an attempt to elucidate the mechanism of GSH synthesis, various isotope exchange experiments have now been carried out with glutathione synthetase. Some other properties and the purification of this enzyme are described in the accompanying paper (3).

EXPERIMENTAL

Materials—P^{32}-ATP was generously supplied by Dr. Eugene Kennedy. P^{32}-ADP was prepared from AMP and radioactive ATP by the action of muscle adenylic kinase (5). Both nucleotides were purified on a Dowex 1 column according to the method of Cohn and Carter (6). The enzyme used, unless stated otherwise, was the preparation which had been purified 5500-fold, as described in the preceding paper (3). The other materials were the same as those used previously (4).

Methods—Hydroxamic acid formation was measured according to the

* Supported by grants-in-aid from the Division of Research Grants and Fellowships of the United States Public Health Service and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago. Some of the data presented in this communication have appeared in preliminary publications (1, 2).

† Present address, Department of Physiological Chemistry, University of California Medical Center, Los Angeles, California.

‡ Present address, Department of Chemistry, Harvard University, Cambridge, Massachusetts.

[1] The following abbreviations are used: AMP, ADP, ATP, adenosinemono-, adenosinedi-, and adenosinetriphosphate, respectively; GSH, glutathione; Pi, orthophosphate; Tris, tris(hydroxymethyl)aminomethane.

method of Lipmann and Tuttle (7). Whenever it was necessary to separate the adenosine nucleotides, Siekevitz and Potter's modification (8) of the Dowex 1 chromatographic technique (6) was employed. For the determination of P^{32}-ATP in the presence of radioactive phosphate, separations were carried out by means of charcoal as described by Crane and Lipmann (9).

Results

It had been observed earlier in this laboratory (10) that ADP is a competitive inhibitor of ATP in GSH synthesis. Since ADP is also a product of the over-all reaction, the inhibitory action of this nucleotide could be

TABLE I

Exchange between ATP and P^{32}-ADP

	ADP	ATP	Exchange
	c.p.m. per μmole	*c.p.m. per μmole*	*per cent*
Complete system	535	405	91
Omit enzyme	2730	32	
" magnesium	2720	30	
Add 0.06 M glycine	524	421	92
" 0.06 " glutamylcysteine	515	494	99

The reaction mixture, 3.0 ml. in volume, contained 0.003 M ATP, 0.00057 M P^{32}-ADP, 0.01 M Tris buffer, 0.01 M KCl, 0.005 M KCN, 0.004 M $MgSO_4$, 3.0 mg. of serum albumin, and 9×10^{-4} mg. of enzyme protein per ml. The incubation was for 60 minutes at 37°, pH 8.3. The nucleotides were separated by Dowex 1 chromatography (8).

taken to indicate a reversal of the process by which ADP is liberated from ATP. Results which are in support of this interpretation have been obtained by incubation of P^{32}-ADP and normal ATP in the presence of low concentrations of GSH synthetase. Incorporation of isotope into ATP took place readily and the exchange of P^{32} between ADP and ATP was essentially complete after 60 minutes (Table I). The exchange of phosphate between ADP and ATP requires magnesium but is not affected in any way by the addition of either glutamylcysteine or glycine. AMP was not formed in this reaction, and, since the *concentrations* of ADP and ATP remained constant under the conditions of the experiment, the observed exchange could not have been due to adenylic kinase. The participation of the latter enzyme was unequivocally ruled out by isolation and degradation of the radioactive ATP (16,000 c.p.m. per μmole) that had been formed in the exchange reaction with P^{32}-ADP. This ATP was added to the GSH synthetase system which splits the nucleotide in accordance with

the equation

$$\text{Glutamylcysteine} + \text{glycine} + \text{ATP} \rightarrow \text{GSH} + \text{ADP} + \text{Pi}$$

The inorganic phosphate which represents the terminal phosphate of ATP was separated from the nucleotides by charcoal and found to contain only 45 c.p.m. On the other hand, if the formation of P^{32}-ATP had resulted from the action of adenylic kinase, the P^{32} concentration in the terminal phosphate of ATP should have accounted for one-half of the total isotope

$$2\text{AMP} \sim P^{32} \rightarrow \text{AMP} + \text{AMP} \sim P^{32} \sim P^{32} \quad (1)$$

in ATP. Since the data in the preceding paper (3) indicate the absence of nucleoside diphosphokinase (11) and in view of the highly purified state of GSH synthetase, it appears probable that the phosphorus exchange between ADP and ATP is indeed catalyzed by the same enzyme which is responsible for GSH synthesis. These findings have previously been accepted as evidence for a reversible phosphorylation of the enzyme by ATP (1, 2), an interpretation which is consistent with the powerful inhibition of GSH synthesis by ADP. Accordingly, it has been postulated that the first step in GSH synthesis from glutamylcysteine is represented by the equation

$$\text{Enzyme} + \text{ATP} \rightleftharpoons \text{ADP} + \text{enzyme-P} \quad (2)$$

If it is correct that GSH synthetase catalyzes the splitting of ATP into ADP and a phosphorylated enzyme, one would not expect an exchange reaction to occur if radioactive orthophosphate instead of ADP^{32} is added to the same system. This had, indeed, proved to be the case. When enzyme and normal ATP were incubated with Pi^{32} alone or in combination with glutamylcysteine, glycine, or both, no significant incorporation of P^{32} into ATP took place.[2] The negative outcome of this experiment confirms earlier observations with the corresponding enzyme from pigeon liver (4).

Since relatively large amounts of the purified enzyme were available, an attempt has been made to test the hypothesis that GSH synthesis involves the formation of a phosphorylated enzyme. 50 mg. of the purified enzyme were incubated, in the presence of magnesium ions, with 20 μmoles of P^{32}-ATP which contained approximately 1×10^6 c.p.m. per μmole in the terminal phosphate group. In order to remove the radioactive ATP remaining at the close of the experiment, the protein was precipitated with 3 volumes of saturated ammonium sulfate, which had been adjusted to

[2] Since, as will be shown later, the synthesis of GSH is a reversible reaction, one would expect P^{32}-orthophosphate to be incorporated into ATP in the presence of glutamylcysteine and glycine. However, under the conditions of the experiment, the concentrations of the products ADP, Pi, and GSH were too small to permit any appreciable synthesis of ATP.

pH 7.0. The protein was redissolved and reprecipitated five more times with ammonium sulfate. After the last precipitation, the protein was found to contain only 200 c.p.m. per mg. If a molecular weight of 100,000 is assumed for the enzyme, the protein would be expected to contain 10,000 c.p.m. per mg. if it contained 1 phosphoric acid residue per molecule of enzyme. This calculation is based on the assumption that the enzyme preparation is pure.

Glycine Exchange—It had been previously concluded that GSH synthetase from pigeon liver was incapable of catalyzing an exchange of the glycine moiety of GSH with free glycine (4). This problem has now been

TABLE II

Exchange of Free Glycine with Glycine Residues of GSH

Addition					Glycine exchanged
ADP	ATP	Arsenate	Phosphate	Mg^{++}	
μmole	*μmole*	*μmoles*	*μmoles*	*μmoles*	*μmole*
1.0		10.0		10.0	0.21
		10.0		10.0	0
1.0				10.0	0
1.0		10.0			0
	1.0	10.0		10.0	0.23
1.0			20.0	10.0	0.07
1.0			50.0	10.0	0.12
			50.0	10.0	0

The reaction mixtures, 1.0 ml., contained 0.1 M Tris buffer, 0.1 M KCl, 0.015 M KCN, 0.01 M $MgSO_4$, 1.0 mg. of GSH, 1.0 mg. of serum albumin, and 0.015 mg. of enzyme. The enzyme was approximately 1500-fold purified. The incubation was for 60 minutes at 37°, pH 8.3. The degree of exchange was determined by measuring the radioactivity of GSH isolated with the aid of a carrier (12).

reinvestigated with the yeast enzyme. Incubation of GSH, C^{14}-glycine, and GSH synthetase does, indeed, result in the formation of C^{14}-GSH, *i.e.* a replacement of the glycine moiety of the tripeptide. As shown in Table II, the exchange reaction requires the presence of magnesium ions and is also dependent upon either ADP or ATP. This requirement for nucleotides cannot be met by AMP. Arsenate is a further component that is necessary, although it can be replaced to some extent by phosphate. As Fig. 1 shows, in the exchange of glycine the nucleotides are effective in extremely low concentrations, indicating that their rôle is a catalytic one.

That the exchange of glycine into GSH is due to the same enzyme that catalyzes GSH synthesis is indicated by the data in Table III. Throughout the purification of the enzyme, the ratio of synthetic to exchange activities has remained constant. Moreover, both reactions proceed op-

timally at pH 8.3. The exchange reaction is only one-tenth as rapid as the synthetic reaction, even under optimal conditions, a finding which may

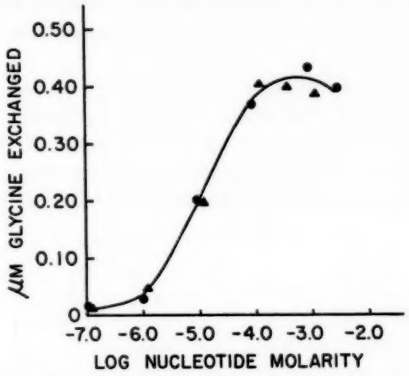


FIG. 1. Effect of ADP (▲) and ATP (●) on glycine exchange. The reaction mixtures, 1.0 ml., contained 0.1 M Tris buffer, 0.1 M KCl, 0.15 M KCN, 0.015 M C^{14}-glycine, 1.0 mg. of serum albumin, 2.0 mg. of GSH, 0.020 M arsenate, 0.010 M $MgSO_4$, and 0.015 mg. of enzyme. The enzyme used was approximately 1500-fold purified. The incubation was for 60 minutes at 37°, pH 8.3.

TABLE III

Synthetic and Exchange Activities of Glutathione Synthetase at Different Stages of Purification

	Specific activity*		Synthesis / Exchange
	GSH synthesis	Glycine exchange	
			per cent
Original autolysate	0.212	0.0186	11.4
Fraction after heat denaturation	13.60	1.28	10.6
Nucleic acid fraction	324.00	28.6	11.4

* Expressed either as micromoles of GSH synthesized or as micromoles of glycine exchanged in 60 minutes per mg. of protein.

explain why previous results obtained with pigeon liver enzyme were negative.

In Fig. 2 is shown the effect of magnesium and manganese ions on the exchange of free glycine with the glycine moiety of GSH. At low concentrations manganese appears to be the more effective of the 2 ions for the exchange reaction. However, at 1×10^{-2} M, which is the optimal concentration for magnesium, the relative effectiveness of the 2 ions is reversed. This is probably attributable to the formation of an insoluble manganese

mercaptide of GSH. In the synthetic reaction, manganese is far less effective than magnesium. Over a concentration range of manganese from 1×10^{-4} to 2×10^{-2} M, the amount of GSH synthesized is never greater than 2 per cent of that observed with the corresponding concentrations of magnesium.

The ability of GSH synthetase to catalyze the exchange of the glycine moiety of the tripeptide recalls the analogous property of glutamine synthetase to catalyze the replacement of the amide of glutamine by free ammonia (13). Since the glutamine-synthesizing enzyme is also capable of exchanging the amide moiety of glutamine for hydroxylamine (13), it

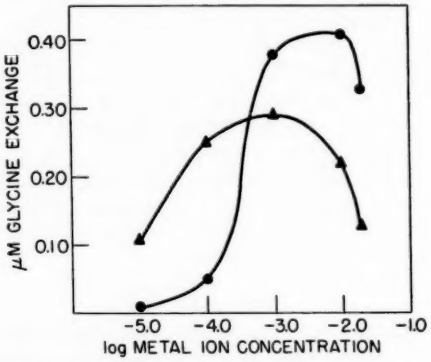


FIG. 2. Effect of magnesium ions (●) and manganese (▲) ions on glycine exchange. The experimental conditions were the same as those given in Fig. 1, except that the incubation mixtures contained 0.001 M ADP. In the experiments in which the manganese concentrations were 2×10^{-3} and 1×10^{-2} M, a precipitate appeared in the reaction mixture.

became of interest to determine whether GSH synthetase could catalyze a similar reaction. That hydroxylamine can indeed serve as a replacement agent for the glycine moiety of GSH is shown by the data in Table IV. As in the exchange reaction with glycine, the replacement of glycine residues by hydroxylamine requires arsenate or phosphate. When phosphate is present, the formation of hydroxamic acid is dependent upon the addition of ADP. However, in the presence of arsenate, ADP, although it stimulates the exchange with hydroxylamine, is not essential. The possibility exists that the hydroxamic acid formation which is apparently ADP-independent is actually promoted by trace amounts of ADP in the enzyme preparation. This, however, is not likely, since the exchange of glycine into GSH in the presence of arsenate did not occur at all unless nucleotide was added even when the enzyme concentration was increased 10-fold over that used in the experiments in Fig. 1. It is evident, there-

fore, that the glycine exchange and the hydroxamic acid formation are not strictly comparable reactions.

The ability of GSH synthetase to promote an exchange of the glycine moiety of GSH suggests that under certain conditions the synthesis of

TABLE IV

Formation of Hydroxamic Acid from GSH

Enzyme	Additions			Hydroxamic acid formed
	ADP	Arsenate	Phosphate	
mg.	*μmoles*	*μmoles*	*μmoles*	*μmoles*
0.05	10.0	20.0		1.01
0.05		20.0		0.37
0.05	10.0			0
0.05	10.0		50.0	0.38
0.05			50.0	0
	10.0	20.0		0

The reaction mixtures, 1.0 ml. in volume, contained 0.1 M Tris buffer, 0.1 M KCl, 0.01 M $MgSO_4$, 0.015 M KCl, 0.013 M GSH, and 0.40 M neutralized hydroxylamine. The incubation was for 60 minutes at 37°, pH 8.3. The hydroxamic acid values are expressed as succinohydroxamic acid equivalents.

TABLE V

Synthesis of ATP from ADP, Pi, and GSH

Enzyme	ADP	GSH	Acid-labile P	ATP synthesized
mg.	*μmoles*	*μmoles*	*c.p.m. per μmole*	*μmole*
	10.0	13.0	19	
0.032	10.0	13.0	511	0.68
0.008	10.0	13.0	296	0.38
0.008		13.0	16	
0.008	10.0		14	

The reaction mixtures, 1.0 ml. in volume, contained 0.1 M Tris buffer, 0.1 M KCl, 0.015 M KCN, 0.01 M $MgSO_4$, 1.0 mg. of serum albumin, and 50 μmoles of P^{32}-orthophosphate (8060 c.p.m. per μmole). The incubation was for 60 minutes at 37°, pH 8.3. The nucleotides were separated from orthophosphate by adsorption on charcoal (9).

GSH may be reversed. The reversibility of glutamine synthesis has recently been demonstrated by Levintow and Meister (14), who have shown that ATP may be synthesized from glutamine, ADP, and Pi. That the over-all reaction glutamylcysteine + glycine + ATP → GSH + ADP + Pi is also a reversible process has been established in the following manner. GSH, ADP, and P^{32}-orthophosphate were incubated under the conditions shown in Table V. The nucleotides were adsorbed on charcoal (9) and

then subjected to hydrolysis in 1 N HCl at 100° for 7 minutes.[3] The acid-labile phosphate was determined and its radioactivity measured. The amount of ATP synthesized was calculated from the equation specific activity of labile phosphate = (specific activity of orthophosphate added × ATP synthesized)/(ADP added + ATP synthesized). The identification of the radioactive nucleotide as ATP was established by adding carrier ATP and separating the nucleotides on a Dowex 1 column. The radioactive component was found to travel with the ATP peak. Furthermore, with the specific activity of labile phosphate as a measure of the amount of ATP formed, the specific activity of the eluted nucleotide, after correction for dilution, differed only by 8 per cent from that of the P^{32}-orthophosphate added to the incubation mixture.[4]

Assuming that equilibrium has been reached in the experiment with the larger amount of enzyme, an equilibrium constant of 1.8×10^4 and a difference of -6040 calories between the standard free energies of hydrolysis of the cysteinyl-glycine bond in GSH and of the terminal pyrophosphate bond of ATP can be calculated. Since it is not certain that in the experiment listed in Table V equilibrium has been attained, the calculated values provide only an approximate measurement of the magnitude of the energy change involved.[5]

DISCUSSION

For the purpose of evaluating the present results in terms of the mechanism of action of GSH synthetase it will be useful to enumerate the various reactions which are promoted by catalytic amounts of the enzyme.

$$\text{Glutamylcysteine} + \text{glycine} + \text{ATP} \rightleftarrows \text{GSH} + \text{ADP} + \text{Pi} \quad (3)$$

$$\text{ATP} + \text{ADP}^{32} \rightleftarrows \text{ATP}^{32} + \text{ADP} \quad (4)$$

[3] To insure the removal of P^{32}-orthophosphate, the charcoal was washed three times with water, two times with 0.02 M normal phosphate buffer, and finally again five times with water.

[4] In a previous attempt (2) to demonstrate the formation of ATP from ADP, Pi, and GSH, the concentration of Pi used was only 5×10^{-4} M. The amount of ATP that could have been formed was too small to detect.

[5] The standard free energy of hydrolysis of a peptide bond in a dipeptide is generally assumed to be -3500 calories (15). There are indications that this value becomes smaller as the length of the peptide chain increases. If the same value is assigned to the cysteinyl-glycine bond of GSH as has been calculated for the hydrolysis of the glycyl-glycine bond of benzoylglycylglycine, namely -2400 calories (15), then the value for the hydrolysis of ATP would be estimated to be about -8500 calories. This is substantially smaller than the generally accepted value of 10,500 calories, but of the same magnitude as that obtained for ATP hydrolysis from a study of the glutamine system by Levintow and Meister (14).

$$\text{GSH} + \text{C}^{14}\text{-glycine} \underset{\text{ADP or ATP}}{\overset{\text{AsO}_4 \text{ or } \text{PO}_4}{\rightleftharpoons}} \text{C}^{14}\text{-GSH} \qquad (5)$$

$$\text{GSH} + \text{NH}_2\text{OH} \xrightarrow[\text{(ADP)}]{\text{AsO}_4 \text{ or } \text{PO}_4} \text{hydroxamic acid} \qquad (6)$$

$$\text{Glutamylcysteine} + \text{hydroxylamine} + \text{ATP} \rightarrow \text{hydroxamic acid} + \text{ADP} + \text{Pi} \qquad (7)$$

Because of the present finding that GSH synthetase catalyzes the synthesis of ATP from ADP and orthophosphate in the presence of GSH, it has now become necessary to postulate that every one of the partial reactions of the over-all process (Reaction 3) can also proceed in the reverse reaction. On the basis of various observations two alternative mechanisms for GSH synthesis have been considered (2). In both of the suggested schemes the initial reaction was assumed to be the reversible phosphorylation of the enzyme by ATP (Reaction 2). For the subsequent steps, Schemes A (Reactions 8 and 9) and B (Reactions 10 and 11) were presented as alternatives.

$$\text{Enzyme-P} + \text{glutamylcysteine} \rightleftharpoons \text{enzyme-glutamylcysteine} + \text{Pi} \qquad (8)$$

$$\text{Enzyme-glutamylcysteine} + \text{glycine} \rightleftharpoons \text{enzyme} + \text{GSH} \qquad (9)$$

$$\text{Enzyme-P} + \text{glutamylcysteine} \rightleftharpoons \text{enzyme} + \text{glutamylcysteine-P} \qquad (10)$$

$$\text{Glutamylcysteine-P} + \text{glycine} \rightleftharpoons \text{Pi} + \text{GSH} \qquad (11)$$

Although the isotope data indicate that the enzyme labilizes the terminal linkage of ATP in such a manner as to permit the free exchange of the ADP moiety, it has not been possible to prove the hypothesis that phosphate becomes concomitantly linked to the enzyme. The present data do not favor the hypothesis (Scheme A) of an enzyme-glutamylcysteine compound as an intermediate. If the formation of this intermediate (Reaction 8) were *reversible*, Pi should be incorporated into ATP in the presence of glutamylcysteine. This was, however, not the case. On the other hand, the failure of glutamylcysteine to inhibit the ATP-ADP exchange can be taken as evidence against the formation of an enzyme-glutamylcysteine by an *irreversible* reaction. Since glycine neither effects the ADP-ATP exchange nor promotes the incorporation of Pi into ATP, the formation of an enzyme-glycine intermediate can be ruled out on the same grounds. On the other hand, the formation of a phosphorylated glutamylcysteine (Reaction 10) is still a possibility, though attempts to isolate this intermediate have met with failure. The formation of this intermediate was previously considered unlikely, since it was believed that the phosphorylation of glutamylcysteine as in Reaction 11 would be ener-

getically improbable. However, the present finding that ATP can be synthesized from ADP, Pi, and GSH removes this objection. To postulate a phosphorylated glutamylcysteine as an intermediate is an attractive hypothesis, since a hydroxamic acid, presumably of glutamylcysteine, can be formed from glutamylcysteine, hydroxylamine, and ATP as well as from GSH and hydroxylamine.

If Scheme B were valid, it should follow that glycine can exchange with GSH, and that a hydroxamic acid is capable of formation from GSH in the absence of ADP. Although the data presented suggest that ADP is not essential for the hydroxamic acid formation, ADP nevertheless stimulates this process markedly, and in the case of the glycine exchange the requirement for either ADP or ATP is absolute. In order to explain the effect of the nucleotides it might be argued that Reaction 11 does occur with rupture of the cysteinyl-glycine bond of GSH, but that the glycine is bound tightly to the enzyme through linkages that are not covalent and that glycine is free to exchange only after complete reversal of the process.

The effect of arsenate in the glycine exchange, as well as in the synthesis of hydroxamic acid from GSH, is interesting in that it implies the formation of arseno compounds of glutamylcysteine and perhaps of ADP. Since both the glycine exchange and hydroxamic acid formation occur more rapidly in the presence of arsenate than of phosphate, it would appear that these arseno compounds are formed more rapidly than the corresponding phosphates, and also that they have considerable stability.

Although the present data are not incompatible with Scheme B, it should be pointed out that the formation of a free phosphorylated glutamylcysteine is difficult to accept if GSH synthetase is indeed a single catalytic entity as the present evidence would seem to indicate.

SUMMARY

Various experiments bearing on the mechanism of the enzymic synthesis of GSH from glutamylcysteine, glycine, and ATP have been carried out with purified GSH synthetase. The enzyme catalyzes an exchange of phosphate between ATP and ADP in the presence of magnesium ions. An exchange of the glycine moiety of GSH for either free glycine or for hydroxylamine is catalyzed by the same enzyme. The synthesis of GSH has been demonstrated to be a reversible reaction.

BIBLIOGRAPHY

1. Snoke, J. E., *J. Am. Chem. Soc.*, **75**, 4872 (1953).
2. Snoke, J. E., and Bloch, K., in Colowick, S. P., Glutathione, New York, 129 (1954).
3. Snoke, J. E., *J. Biol. Chem.*, **213**, 813 (1955).
4. Snoke, J. E., Yanari, S., and Bloch, K., *J. Biol. Chem.*, **201**, 573 (1953).

5. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **148,** 117 (1943).
6. Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72,** 4273 (1950).
7. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159,** 21 (1945).
8. Siekevitz, P., and Potter, V. R., *J. Biol. Chem.*, **200,** 187 (1953).
9. Crane, R. K., and Lipmann, F., *J. Biol. Chem.*, **201,** 235 (1953).
10. Yanari, S., Snoke, J. E., and Bloch, K., *J. Biol. Chem.*, **201,** 561 (1953).
11. Berg, P., and Joklik, W. K., *Nature*, **172,** 1008 (1953).
12. Johnston, R. B., and Bloch, K., *J. Biol. Chem.*, **188,** 221 (1951).
13. Elliott, W. H., *J. Biol. Chem.*, **201,** 661 (1953).
14. Levintow, L., and Meister, A., *J. Biol. Chem.*, **209,** 265 (1954).
15. Borsook, H., *Advances in Protein Chem.*, **8,** 127 (1953).

SOME NOTES ON THE EXCHANGE OF IODINE WITH THYROXINE HOMOLOGUES

By GEOFFREY I. GLEASON

(From the Abbott Laboratories, Oak Ridge Division, Oak Ridge, Tennessee)

(Received for publication, September 21, 1954)

A number of methods have been employed for the preparation of thyroxine labeled with I^{131}. Frieden, Lipsett, and Winzler (1) described in their summary an exchange procedure which was developed as a result of the studies of Miller *et al.* (2) on the iodotyrosines. Since this route offers the most practical approach to labeling with short lived, radioactive iodine (I^{131}) in high yield, we have directed our efforts toward improving the technique and resolving some of the uncertainties mentioned in the original work. The more important findings are reported here.

It was pointed out by Miller *et al.* (2) that compounds capable of reacting with iodine inhibited exchange of radioactive iodide with diiodotyrosine and the suggestion was made that the active agent was actually iodine. We have verified this in connection with labeled thyroxine which shows no exchange on contact with large amounts of inactive iodide provided sufficient amounts of sulfite ion, or other reducing agent, are present to insure the absence of free iodine.

The thyroxine-iodide exchange reported by Frieden *et al.* (1) involved somewhat drastic reaction conditions and the liberation of some free iodine at increased temperature to "catalyze" the process is indicated. By comparison, the conditions which we provide for the exchange of labeled iodine with thyroxine are exceedingly mild. At pH 7 to 8, the reaction proceeds rapidly to equilibrium at room temperature in *tert*-butyl alcohol solution at concentrations of 10^{-4} M. The recently identified, biologically active compound, 3,5,3′-triiodothyronine (3), behaves similarly.

Procedures

Our work was all performed at the micro level with analysis carried out by means of ascending paper chromatography. One of the better solvents for resolving the various compounds encountered was *tert*-amyl alcohol saturated with 2 N ammonium hydroxide. R_F values for the system are given in Table I.

Fig. 1 shows a mechanically recorded graph of such a chromatogram. Scanning is complete within 15 minutes and the results are quickly interpreted by determination of the areas with a planimeter. The example illustrated shows the reaction of 0.50 μmole of I_2 with 1.0 μmole of triiodo-

XUM

thyronine (ThI_3) after substitution is complete. Within the experimental error, the distribution of the radioactivity follows the molar ratio of the

$$0.5I_2 + ThI_5 \rightarrow 0.5ThI_4 + 0.5ThI_3 + 0.5I^-$$

products. This finding has been verified by numerous reactions at other

TABLE I

Approximate R_F Values for tert-Amyl Alcohol-NH_4OH Solvent

Substance	R_F
Iodate	0.00
Iodotyrosines	0.10
Iodide	0.20
Thyroxine	0.34
Triiodothyronine	0.54
Diiodothyronine	0.58

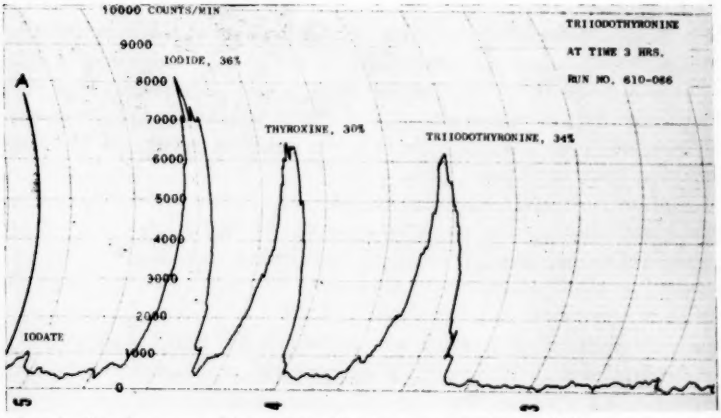


FIG. 1. Graph prepared by automatic scanner showing activity on paper chromatogram. Development from left to right.

molar ratios and in reactions involving thyroxine and triiodothyronine simultaneously.

Chromatographic evaluation of the reaction mixture resulting from 0.50 μmole of I_2 and 1.0 μmole of 3,5-diiodothyronine showed only labeled thyroxine and triiodothyronine. Carrier diiodothyronine, located by the diazotized sulfanilic acid spray test, had migrated slightly but definitely ahead of the triiodothyronine zone and was completely inactive.

The course of the exchange was also followed by this procedure: To 1.5

ml. of water containing tracer I^{131} and 0.50 μmole of I_2 were carefully added 2.0 ml. of *tert*-butyl alcohol to form an insulating layer. 1.0 μmole

TABLE II

Status of Reaction of 1.0 μmole of Triiodothyronine with 0.50 μmole of I_2 with Time

Substance	Per cent present at					Theoretical*
	10 sec.	5 min.	10 min.	15 min.	3 hrs.	
Iodate					Trace	
Iodide	55	45	43	41	36	33
Thyroxine	5	17	22	25	30	33
Triiodothyronine	40	38	35	34	34	33

* Theoretical yield at equilibrium after substitution, if possible, is complete.

TABLE III

Status of Reaction of 1.0 μmole of Thyroxine with 0.50 μmole of I_2 with Time

Substance	Per cent present at					Theoretical*
	10 sec.	5 min.	10 min.	15 min.	3 hrs.	
Iodate	4	5	7	8	18	
Iodide	46	41	41	39	46	50
Thyroxine	50	52	48	48	20	50
Triiodothyronine						
Fragment 1		2	4	5	10	
" 2				Trace	6	

* Theoretical yield at equilibrium after substitution, if possible, is complete.

TABLE IV

Status of Reaction of 1.0 μmole of Diiodothyronine with 0.50 μmole of I_2 with Time

Substance	Per cent present at			
	10 sec.	5 min.	10 min.	3 hrs.
Iodate		1	2	8
Iodide	85	65	59	46
Thyroxine	3	10	12	17
Triiodothyronine	12	24	27	29
Diiodothyronine				

of triiodothyronine in 2.0 ml. of the same solvent was then cautiously admitted without disturbing the layers. The reaction was initiated by thorough agitation of the system while at the same time the pH was ad-

justed to the range of 7 to 8 with 4 drops of saturated Na_2HPO_4 buffer. Immediately upon mixing (5 to 10 seconds), a sample was withdrawn and "quenched" by adding to a small volume of wet *tert*-butyl alcohol containing sufficient sulfite ion to reduce the free iodine and arrest the reaction at whatever stage it had reached. Subsequent samples were taken at definite intervals and similarly treated. The chromatographic data from these samples are given in Table II. Tables III and IV summarize the same type of study with thyroxine and diiodothyronine, respectively.

DISCUSSION

The data from Table II show that the rate of exchange of iodine with triiodothyronine materially exceeds that of iodination to thyroxine. Exchange with thyroxine also proceeds at a comparable speed, which accounts for the decrease in labeled triiodothyronine as thyroxine is produced.

The molar distribution of radioactivity in the products is somewhat surprising and implies that only 1 iodine atom in a given molecule is subject to exchange. This is in agreement with the comment of Larson *et al.* (4), although reaction conditions and molar ratios are at variance. The true explanation stems from a consideration of the actual number of I^{131} atoms compared with the number of thyronine molecules and iodine (I^{127}) molecules present in the usual reaction. This ratio is approximately 1:1000, even when preparations at the millicurie level are made, and it follows that the probability of multilabeling is extremely remote. The exchange reaction is, of course, completely reversible and isotopic equilibrium is reached when molar specific activities are constant, regardless of the number of exchangeable atoms present in a component.

In the above connection, the failure of 3,5-diiodothyronine to participate in iodine exchange leads to the conclusion that only the "prime" positions (ortho to the hydroxy group) are involved in the labeling of thyroxine and triiodothyronine by this method.

Insufficient data have been obtained to permit a clear statement concerning the mechanism by which these exchange equilibria are so rapidly established. The rate, under such mild conditions, suggests resemblance to the iodine-iodide exchange which proceeds through the formation of an intermediate complex ion

$$I_2 + I^- = I_3^-$$

When electrophilic substitution is possible (as with triiodothyronine), I_2 is simultaneously utilized in this process and soon removed from the field of action. Otherwise (as with thyroxine), only exchange occurs followed by the gradual hydrolysis of iodine to the iodate according to the well

known reactions

$$I_2 + OH^- = HOI + I^-$$
$$HOI = H^+ + IO^-$$
$$3IO^- \rightarrow IO_3^- + 2I^-$$

The oxidizing power of hypoiodous acid could well account for the observed degradation of the labeled thyroxine on prolonged contact with the system (Table III).

The quantitative aspect of these exchange reactions with complete evaluation via paper chromatography suggests an extension of the analytical technique for the microdetermination of thyroxine (4). We have already applied this in connection with estimations of the specific activity of our labeled preparations. Useful biological assays at the submicro level should be possible with a minimum of labor and ultimate selectivity.

The possibility of these reactions occurring *in vivo* has not been examined.

SUMMARY

1. Iodide does not exchange with iodinated thyronines under conditions assuring the absence of free iodine.

2. Iodine exchanges readily with both thyroxine and triiodothyronine in synthetic solution at physiological pH.

3. The rate of this exchange at 10^{-4} M concentration far exceeds that of substitution under the same conditions.

4. The distribution of radioactivity is proportional to the molar ratios of the products, *i.e.* quantitative.

5. Labeling by exchange involves only the "prime" positions.

6. A new solvent is described which provides excellent chromatographic resolution of the compounds encountered.

BIBLIOGRAPHY

1. Frieden, E., Lipsett, M. B., and Winzler, R. J., *Science*, **107**, 353 (1948).
2. Miller, W. H., Anderson, G. W., Madison, R. K., and Salley, D. J., *Science*, **100**, 340 (1944).
3. Gross, J., and Pitt-Rivers, R., *Biochem. J.*, **53**, 645 (1953).
4. Larson, F. C., Coulson, D. M., and Albright, E. C., *J. Biol. Chem.*, **196**, 45 (1952).

CONVERSION OF MONOSACCHARIDES TO SUCROSE AND CELLULOSE IN WHEAT SEEDLINGS*

By J. EDELMAN,† V. GINSBURG, AND W. Z. HASSID

(From the Department of Plant Biochemistry, College of Agriculture, University of California, Berkeley, California)

(Received for publication, October 4, 1954)

The conversion of monosaccharides to sucrose in the dark has long been studied by introducing solutions of hexoses into leaves or fragments of plant organs. It has been established that sucrose can be synthesized *in vivo* in plants at the expense of monosaccharides such as glucose, fructose, or mannose (1). This observation has been recently confirmed with randomly C^{14}-labeled monosaccharides (2–4). When radioactive glucose or fructose was infiltrated into *Canna* leaf tissue (2), sucrose appeared to be readily formed from either monosaccharide. Analysis of the glucose and fructose derived from the hydrolysis of the sucrose showed that both monosaccharide moieties were labeled. These investigations did not reveal, however, whether the monosaccharide introduced into the plant is directly incorporated into the sucrose molecule or whether it is first degraded to shorter chain compounds. The present paper reports further work on this problem in which glucose and fructose labeled in known positions were used.

Glucose-1-C^{14} was recently used by Greathouse (5) and by Brown and Neish (6) to investigate the synthesis of cellulose in plants. The experiments described by these workers were performed under conditions of slow rate of substrate absorption which necessitated long periods of incubation. Greathouse isolated the cellulose from cotton bolls 90 days after injection of labeled glucose, while Brown and Neish isolated the polysaccharide from wheat plants 24 to 96 hours after injection.

In the present investigation actively metabolizing wheat seedlings, 3 days old, were used. Absorption of the substrate by the seedlings was rapid and its transformation to other carbohydrates occurred readily; hence short periods of incubation (order of minutes) were adequate. C^{14}-labeled monosaccharide gave rise to labeled sugar phosphates, sucrose, and cellulose in the tissue. These compounds were separated, their mono-

* This work was supported in part by a research contract with the United States Atomic Energy Commission.

† Fellow of the Rockefeller Foundation, 1953–54. Present address, Research Institute of Plant Physiology, Imperial College, South Kensington, London, S. W. 7, England.

saccharide constituents were degraded, and the distribution of C^{14} in the molecules was determined.

Materials and Methods

Radioactive Sugars—Uniformly C^{14}-labeled glucose was prepared by Dr. E. W. Putman (7) of this laboratory. The sugar was chromatographically pure and had activity of about 30 μc. per mg.

Glucose-1-C^{14} was prepared by an adaptation (8) of the method of Isbell *et al.* (9). It was chromatographically pure and had activity of about 7.5 μc. per mg. Degradation of the sugar (see below) showed that all the activity resided in carbon atom 1; the activity in the rest of the molecule was less than 1 per cent of the total.

Glucose-6-C^{14} was obtained from Dr. H. S. Isbell (National Bureau of Standards). Its activity was about 1 μc. per mg.

Fructose-1-C^{14} was prepared by enolization of glucose-1-C^{14} (8).

The labeled sugars were diluted with anhydrous glucose or fructose (analytical grade) when necessary.

Estimation of Radioactivity—Samples were counted directly on Whatman No. 1 filter paper, with a Tracerlab rate meter (SU-3A) supplied with a Geiger tube (2). This instrument can be read with an accuracy of ±5 per cent of the full scale deflection in ranges of 200 to 20,000 c.p.m. More accurate estimations were made on barium carbonate samples or sugar films mounted on aluminum disks, 2.5 cm. in diameter, by means of standard end window Geiger counting equipment. Unless otherwise stated radioactivity measurements are expressed as mean values of duplicate samples corrected for self absorption, daily counter variation, and background.

Sugar Analysis—Reducing sugar was estimated by the method of Somogyi (10) as modified by Nelson (11). Fructose was determined either by the modified method of Roe (12) or by that of Roe *et al.* (13).

Preparation of Wheat Seedlings—Wheat grain, variety Big Club, which was previously stored in air at room temperature, was soaked in tap water overnight, spread on moist filter paper, and left in the dark. After 3 days, at which time the coleoptiles were about 2 cm. long and three roots were normally present, each seedling was removed from the grain and placed in tap water through which a rapid stream of air was passed. Aeration was continued for 2 to 4 hours, the water being frequently changed. Preliminary experiments with randomly labeled glucose-C^{14} indicated that seedlings separated from the grain and used without the ensuing period of aeration showed an initial release of reducing material, followed by a lag in sugar uptake before the maximal absorption rate was reached. Seedlings which had been separated and aerated absorbed sugar at a constant

rate for several hours. The rate of absorption by seedlings attached to the grain was relatively low.

Dry weight was estimated on tissue dried in an air oven at 80° for 24 hours.

Incubation with C^{14}-Labeled Glucose—In preliminary experiments the seedlings were suspended in 1 per cent glucose solution; in later experiments 0.1 per cent solutions were used. The tissue suspension was vigorously aerated during incubation.

In a typical experiment, thirty seedlings (dry weight about 4 mg. per seedling) were placed in 4 ml. of the radioactive sugar solution in a vessel of 20 ml. capacity with a sintered glass bottom through which air was blown. After appropriate time intervals the seedlings were removed, washed in rapidly running tap water for at least 30 seconds, plunged into about 20 ml. of boiling 70 per cent ethanol, and maintained at boiling point for 5 minutes. The ethanol extract was decanted.

Extraction and Separation of Ethanol-Soluble Components—After repeating the extraction of the tissue with three further portions of 70 per cent ethanol, the extracts were combined, filtered, and evaporated to a small volume on a steam bath and the concentrated solution was (about 5 per cent sugar) applied as an even band 17 inches long on a filter paper sheet (Whatman No. 1, 18 × 24 inches) parallel to the longer edge. The paper sheet was developed in butanol-acetic acid-water mixture (14) for 3 days and the radioactive material located by exposing the dried sheet to Eastman Kodak "no screen" x-ray film. The appropriate bands were cut from the chromatogram and eluted with water and the products dried *in vacuo* over calcium chloride.

Preparation of Samples for Degradation—Because of the substrate specificity requirements of the organism used in the sugar degradation, it was necessary to convert all the labeled carbohydrate samples to free glucose.

Sucrose was first completely hydrolyzed to glucose and fructose with invertase (British Drug Houses invertase concentrate); the monosaccharides were separated chromatographically on paper sheets, located, and eluted as previously described. The fructose fraction was epimerized to glucose by heating (with or without fructose carrier) in a sealed tube in 0.3 ml. of 0.2 M sodium phosphate buffer, pH 7.5, at 100° for 2 hours, after which time the resulting mixture was resolved chromatographically on paper. There is evidence that no randomization of the hexose chain occurs during the epimerization process (15). The yield of glucose was about 10 per cent.

The hexose phosphates were resolved into two main bands on one-dimensional chromatograms with butanol-acetic acid-water as solvent. The bands were eluted and hydrolyzed by incubation with phosphatase from

General Biochemicals, Inc. (0.4 per cent) at pH 8.0 for 20 hours at 35° in the presence of toluene. One of the resulting solutions contained radioactive glucose and the other both glucose and fructose.

The residual tissue obtained after extracting the wheat seedlings with ethanol contained chiefly cellulose and hemicellulose. The tissue was freed from hemicellulose by stirring twice with 1 N sodium hydroxide for 24 hours. The residue was exhaustively washed with water, heated with 1 N sulfuric acid in a sealed tube for 6 hours at 100°, washed, and blotted between filter papers. Glucose was obtained by hydrolysis of this purified cellulose by adaptation of the procedure of Monier-Williams (16): The material was treated with 72 per cent sulfuric acid for 7 days at room temperature, and the resultant dark suspension was diluted to 0.7 per cent sulfuric acid, filtered, and refluxed for 15 hours. After cooling, the acid was titrated with barium hydroxide, with phenolphthalein as an indicator, and the barium sulfate precipitate was removed by filtration. The solution was concentrated to a sirup *in vacuo*, made up to suitable volume, and chromatographed. Glucose was the major radioactive constituent. The yield of chromatographically pure glucose was approximately 21 per cent, based on the tissue residue obtained after ethanol and alkali extraction.

Degradation of Glucose—The method used for degradation of glucose was essentially that of Gunsalus and Gibbs (17). The active glucose was diluted with carrier to give a total of 0.5 mmole of sugar and dissolved in water (1 to 5 ml.) and about a 10 per cent portion was taken for measurement of total activity, which was obtained after complete oxidation to CO_2 by the persulfate method of Katz *et al.* (18). The remainder was subjected to heterolactic fermentation by *Leuconostoc mesenteroides* (17), which degrades glucose as follows:

$$\underset{}{C_6H_{12}O_6} \rightarrow \underset{(1)}{CO_2} + \underset{(2)\ (3)}{CH_3 \cdot CH_2OH} + \underset{(4)\ (5)\ \ (6)}{HOOC \cdot CHOH \cdot CH_3}$$

Incubation with the organism was carried out in sodium phosphate solution at pH 6 at about 2 cm. pressure. Conditions were arranged so that the fermentation was complete in less than 1 hour. After incubation the cells were immediately removed by centrifugation.

The ethanol was obtained by distillation of the reaction mixture. Usually it was sufficient to obtain the sum activity of carbon atoms 2 and 3 in which case the ethanol was oxidized directly with persulfate. When an estimate of the activity in each atom was required, the ethanol was oxidized in the presence of 100 mg. of potassium dichromate and 12 N sulfuric acid to acetic acid; the acetic acid was recovered by steam distillation and split to CO_2 (C-3) and methylamine (C-2) with sodium azide and sulfuric

acid (19). Methylamine was oxidized to CO_2 with alkaline potassium permanganate (19).

The residual fermentation mixture after removal of the ethanol was concentrated to about 1 ml. and taken up with 20 gm. of fused potassium hydrogen sulfate and the lactic acid eluted from a column of the resultant dry powder with ether. The ether was removed, and the lactic acid was oxidized with acid permanganate in aqueous solution (19) to yield CO_2 (C-4), and acetic acid (C-5 and C-6) which was either directly oxidized by persulfate or further degraded as described above. CO_2 was collected in standard sodium hydroxide and converted to barium carbonate and activity measurements were made on about 10 mg. samples mounted on aluminum disks (diameter, 2.5 cm.). Reagent blank values, which were rarely as high as 5 per cent for 0.5 mmole samples of glucose, were determined at all stages.

Reliability of Method—With 0.5 mmole quantities of glucose the sums of activity of the individual carbon atoms determined by the microbiological method and the total activity obtained from persulfate oxidation agreed well. Degradation of uniformly C^{14}-labeled glucose by the biological method gave values ranging from 15.2 to 16.7 per cent per carbon atom (theoretical, 16.7 per cent), with a total recovery of 97.2 per cent. Degradation of glucose-1-C^{14} by the same method showed that all the activity resided in C-1 (experimental value, 102 per cent). The sum of activity in the other 5 carbon atoms was about 1 per cent, the total recovery of activity being 103.1 per cent.

It was found that, when 0.1 mmole quantities were subjected to degradation, recoveries were consistently lower (80 to 85 per cent), possibly owing to unavoidable contamination by atmospheric CO_2 and other reagent blank values which become significant at this level.

Results

Distribution of C^{14} in Sucrose, Cellulose, and Hexose Phosphates—Analysis of wheat seedlings before incubation with sugar solutions revealed the following results: shoots, 1.6 per cent sucrose, 4.6 per cent fructose, and 2.4 per cent glucose; roots, 1.5 per cent sucrose, 1.9 per cent fructose, and 3.2 per cent glucose, calculated on the dry weight of the tissue.

Chromatographic analysis of the external medium, before and after incubation of the wheat seedlings with glucose-1-C^{14}, showed that glucose was the only active component; no other sugars could be detected. The ethanol extract of the tissue after incubation contained about 45 per cent of the dry weight of the seedlings. Paper chromatography showed that most of the activity of this extract resided in the sucrose fraction with some in the sugar phosphates and very little in the monosaccharides.

In the experiment described in Table I resolution of the sucrose to its monosaccharide constituents showed that the glucose moiety (2.10 mg.) contained a total activity of 1.61×10^5 c.p.m. (counted as sugar film), while the fructose moiety (2.24 mg.) contained a total of 1.44×10^5 c.p.m.

The results of the distribution of C^{14} in the hexoses derived from sucrose and cellulose are presented in Table I. The data show that the percentage incorporation of C^{14} activity in C-1 of the monosaccharide moieties of the sucrose and in the glucose of the cellulose was practically identical (78 per cent). The values for C^{14} activity in C-6 of the hexose units of these compounds were also approximately the same (15 per cent). Examination of the distribution of C^{14} between C-1 and C-6 of the hexose units of sucrose,

TABLE I

Per Cent Distribution of C^{14} in Hexoses Derived from Complex Saccharides after Incubating Wheat Seedlings with Glucose-1-C^{14}

50 seedlings were incubated for 30 minutes in 4 ml. of medium containing 3 mg. of glucose-1-C^{14} with specific activity of 7.5 μc. per mg. The values were obtained by *L. mesenteroides* degradation and are expressed as percentages of total activity in $BaCO_3$ resulting from persulfate oxidation of the sugar sample.

Carbon atom	Glucose moiety of sucrose	Fructose moiety of sucrose	Glucose from cellulose
1	78.0	77.8	78.0
2 + 3	1.9	1.7	
4	0.5	2.5	3.3
5	0.0		0.0
6	15.6	15.3	14.5
Total recovery.....	96.0	97.3	95.8

hexose phosphates, and cellulose as a function of time showed a decrease in activity of C-1 with a corresponding increase in C-6 during the first 15 minutes. After that time the ratio of activity in the 2 carbons remained practically constant at least up to 2 hours (Table II).

Continuous incubation of wheat seedlings for 8 hours with uniformly C^{14}-labeled glucose resulted in the distribution of C^{14} in glucose from cellulose shown in Table III. Glucose-1-C^{14} gave similar results to those shown in Table I, except that there was a rather greater randomization between C-1 and C-6 during the longer period; the effect of incubation with glucose-6-C^{14} was to randomize C-1 and C-6 approximately to the same extent, but in inverse ratio.

Distribution of C^{14} in Glucose Moiety of Sucrose When Incubated with Fructose-1-C^{14}—An experiment in which fructose-1-C^{14} was used as substrate (70 seedlings incubated for 2 hours in 10 ml. of solution containing

8 mg. of fructose-1-C^{14}; total activity 6.5 μc.) gave similar results to those already described with glucose-1-C^{14}. Thus the glucose moiety of sucrose

TABLE II

Change in Distribution of C^{14} in C-1 and C-6 of Hexose with Time after Continuous Incubation of Wheat Seedlings with Glucose-1-C^{14}

50 seedlings were used for each of 8 and 15 minute samples; twenty for others. Incubation was carried out in 5 ml. of medium containing 3 mg. of glucose-1-C^{14} with specific activity of 7.5 μc. per mg.

Incubation time	C^{14} ratio, $\frac{C\text{-}1}{C\text{-}1 + C\text{-}6} \times 100$			C^{14} ratio, $\frac{C\text{-}6}{C\text{-}1 + C\text{-}6} \times 100$		
	Glucose from sucrose	Fructose from sucrose	Glucose from hexose phosphates	Glucose from sucrose	Fructose from sucrose	Glucose from hexose phosphates
min.						
8	86	90	84	14	10	16
15	78	78	80	22	22	20
60	77	78	78	23	22	22
120	74	77	78	26	23	22

TABLE III

Per Cent Distribution of C^{14} in Glucose from Cellulose after Incubation of Wheat Seedlings with Uniformly C^{14}-Labeled Glucose, Glucose-1-C^{14}, and Glucose-6-C^{14}

In each experiment, forty-one seedlings were incubated for 8 hours in 4 ml. of medium containing 15 mg. of labeled glucose of total activity as follows: 15 μc. of uniformly C^{14}-labeled glucose, 25 μc. of glucose-1-C^{14}, 1 μc. of glucose-6-C^{14}, respectively.

Carbon atom	Evenly labeled C^{14}-glucose	Glucose-1-C^{14}	Glucose-6-C^{14}
1	16.0	70.9	17.1
2 + 3	34.6	4.0	9.1
4	17.1	2.2	0.8
5	18.3	0.2	0.0
6	17.5	22.3	72.2
Total recovery....	103.5	99.6	99.2

showed 71 per cent of the activity in C-1 and 15 per cent in C-6. The rest of the carbon chain contained negligible activity; total recovery was 89 per cent.

Because of the limited C^{14} activity in the sucrose, enolization of the fructose component to glucose was not practicable. The distribution of C^{14} in the fructose moiety was therefore not determined.

Glucose-6-phosphate-Fructose-6-phosphate Interconversion in Vitro—Wheat seedlings were grown in the usual manner, separated from the grain, and washed thoroughly with tap water. The tissue (25.5 gm. wet weight) was ground in a mortar and the homogenate was squeezed through muslin to yield 17 ml. of extract. The extract was centrifuged, dialyzed against four changes of tap water at 3° for 45 hours, and stored frozen at −15°.

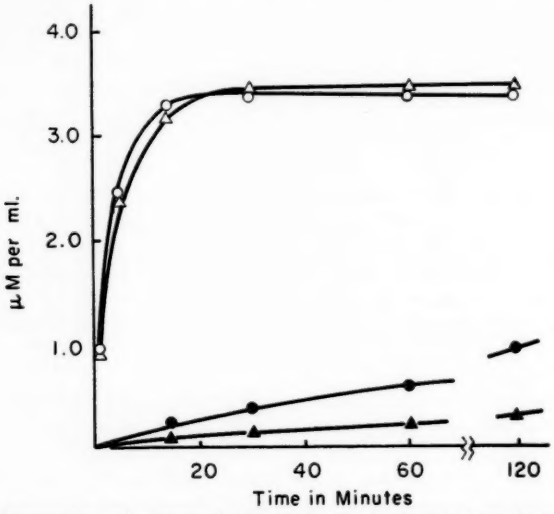


FIG. 1. Conversion of glucose-6-phosphate to fructose-6-phosphate by wheat seedling extract. ○ represents appearance of fructose-6-phosphate when no NaF was added; △, when NaF was added. ● represents appearance of inorganic phosphate when no NAF was added; ▲, when NaF was added. The solutions contained, per ml., 13.5 μmoles of glucose-6-phosphate, 70 μmoles of triethanolamine-Versene buffer, pH 7.1, 0.125 ml. of dialyzed wheat seedling extract and, when present, 50 μmoles of NaF. Incubation carried out at 30°. Samples (0.5 ml.) delivered into 6 per cent trichloroacetic acid (2.0 ml.) at appropriate time intervals.

The concentration of protein in the extract was 2.6 mg. per ml. This extract was used to demonstrate the presence of hexosephosphate isomerase by the appearance of fructose when the extract was allowed to act on glucose-6-phosphate.

A solution containing glucose-6-phosphate, wheat seedling extract, and buffer, pH 7.1, was incubated at 30°. Analysis of the extract showed that fructose appeared rapidly and reached a maximum after 30 minutes (Fig. 1), at this time the proportions of ketose and aldose (calculated as the 6-phosphates) being 25.5 and 74.5 per cent, respectively. This ratio, which agrees with the known equilibrium in the presence of phosphohexose

isomerase (20), remained constant during a further 90 minutes incubation. The initial velocity, calculated over the first 5 minutes, showed that 0.4 μmole of fructose-6-phosphate (about 12 per cent of the final concentration) appeared per minute per ml. The results were essentially the same in the presence or absence of 50 μmoles of NaF per ml. of solution. The liberation of inorganic phosphate was slow (0.4 μmole per ml. in the first 30 minutes), and this was considerably reduced (to 0.14 μmole per ml.) in the presence of NaF. There was no significant pH optimum in the range investigated, pH 6.5 to 8.0.

DISCUSSION

The results obtained with wheat seedlings are in agreement with those previously obtained in this laboratory with *Canna* leaf disks (2), showing that glucose and fructose taken up by the tissue do not enter the monosaccharide pool in the plant but may become phosphorylated at the time they enter the cells. The major proportion of the C^{14} taken up in the form of labeled monosaccharide appeared in sucrose and the sugar phosphates; in contrast to the experiments with *Canna* leaf disks comparatively little activity was incorporated into other ethanol-extractable compounds. A small proportion of the activity after feeding glucose-1-C^{14} was recovered in glucose separated from the hydrolysis products of cellulose.

In the experiments with wheat seedlings and glucose-1-C^{14}, degradation of the glucose and fructose moieties from sucrose and the glucose constituent of cellulose and of hexose phosphates showed that the major proportion of activity resided in C-1, indicating that hexose residues may be directly incorporated into the complex saccharides. However, considerable randomization had occurred between carbon atoms 1 and 6 in the combined hexoses in the shortest time intervals (8 minutes for soluble sugars (Table II), 30 minutes for cellulose (Table I)). Little activity could be detected in carbon atoms 2 to 5 even after longer periods of incubation (2 hours for sucrose and hexose phosphates, 8 hours for cellulose). Thus the data show that approximately 9 to 15 per cent of the C^{14} label was incorporated into C-6 during the first 8 minutes, increasing to some 20 per cent during the following 7 minutes. There was little subsequent change in the ratio of labeling in C-1 and C-6. Our results of randomization between C-1 and C-6 in cellulose agree with those of Brown and Neish (6), but are in contrast with those of Greathouse (5) who could find no randomization in glucose obtained from cellulose from cotton bolls for as long as 90 days after feeding glucose-1-C^{14}.

The activity in C-6 may have been derived from the incorporation of a certain proportion of hexose which had been synthesized through randomization of C-1 and C-6 via reversible functioning of part of the glycolytic

pathway, involving recombination of the two triose phosphates. Although the per cent activity in C-6 may give a rough measure of this process, it is possible that complete equilibration between the triose phosphates does not occur and consequently the proportion of the total combined hexose cycled through this pathway may be appreciably greater than is indicated. However, in view of the diminution of activity in C-1 and the corresponding increase of activity in C-6 during the initial time periods (Table II), it is unlikely that all the hexose units constituting the complex saccharides are derived via this pathway.

An alternative pathway for randomization of these 2 carbon atoms in the combined hexose may involve products derived from the monophosphate shunt (21). In this scheme C_3 fragments labeled at C-3, derived from glucose-1-C^{14} via glycolysis, would serve as acceptors of unlabeled C_3 fragments transferred from sedoheptulose by transaldolase, thus forming fructose-6-phosphate labeled at C-6. However, this mechanism could not give rise to labeling in C-1 with glucose-6-C^{14} as substrate; thus the finding that C-1 and C-6 in the glucose of cellulose are randomized to the same extent (in inverse ratio) when glucose labeled at C-1 or C-6 is given appears to preclude such a pathway.

It is interesting to note that in animals the administration of glucose-1-C^{14} gives rise to glycogen (22) or lactose (23) in which the hexoses are labeled almost exclusively in C-1, there being no indication of significant randomization between C-1 and C-6 after several hours. With the same substrate *Acetobacter xylinum* forms cellulose in which the glucose is labeled in carbons 1, 3, and 4, with the major proportion located at position 1 (24).

Comparison of the labeling of the monosaccharide moieties of the sucrose formed from the glucose-1-C^{14} showed that the specific activity of the glucose was approximately 10 per cent higher than that of the fructose. In experiments with *Canna* leaf disks (2) the specific activity of the glucose moiety of the sucrose derived from the infiltrated radioactive glucose was approximately 50 per cent greater than that of the fructose, and, correspondingly, when labeled fructose was infiltrated, the reverse was observed. The smaller difference of C^{14} label in the two monosaccharide moieties of sucrose in wheat seedlings may be explained by the assumption that in this actively metabolizing tissue a more rapid equilibrium is established between the glucose and fructose precursors derived from the glucose-1-C^{14}. This equilibration could be achieved by phosphohexose isomerase activity which was demonstrated in the tissue extracts. From initial velocities derived under the experimental conditions used for measurements of this activity, it was calculated that extracts were able to produce an amount of fructose-6-phosphate equivalent to that of the

fructose moiety of sucrose synthesized by a corresponding amount of tissue at a rate roughly 10 times faster than that of glucose uptake by the seedlings.

Since the distribution of activity among the individual carbon atoms in the glucose and fructose moieties of sucrose and the glucose constituent of cellulose is nearly identical, it is probable that the monosaccharide constituents of these higher saccharides share a common precursor.

Two mechanisms of sucrose formation are known at present to occur *in vitro*, *viz.* those of *Pseudomonas saccharophila* (25) and wheat germ (26). In both a glucose donor (glucose-1-phosphate or uridine diphosphate glucose, respectively) combines with free fructose to form sucrose with the elimination of phosphate or uridine diphosphate. As the free fructose in the plant never becomes significantly labeled, the sucrose would be expected to be labeled predominantly in the glucose moiety, should this type of mechanism be acting in wheat seedlings. Thus, our finding that both moieties of sucrose are similarly labeled would indicate that free hexose is not involved in its synthesis; nevertheless, the possibility of utilization of free radioactive monosaccharide formed in transient amounts at the site of sucrose synthesis cannot be entirely overlooked.

SUMMARY

The transformation of C^{14}-labeled glucose and fructose was studied in actively metabolizing wheat seedlings during periods ranging from 8 minutes to 8 hours. The radioactive sugar was rapidly absorbed, giving rise to labeled hexose phosphates, sucrose, and cellulose. The sucrose was always approximately equally labeled in both moieties.

When seedlings absorbed glucose-1-C^{14}, C-1 of the glucose and fructose isolated from the sucrose and of the glucose from cellulose was found to contain the major proportion of activity (78 per cent after 30 minutes). C-6 contained virtually all the remainder (15 per cent), which may have been produced through breakdown of the hexose molecule to trioses and randomization of C-1 and C-6. The intermediate carbons in the chains (C-2 to C-5) contained very little activity (less than 5 per cent); glucose from the hexose phosphate fraction gave comparable results. A similar distribution was found in the glucose moiety of sucrose after absorption of fructose-1-C^{14}.

Randomization between C-1 and C-6 in the glucose and fructose moieties of the sucrose and in the glucose constituent of the cellulose showed little change with time.

The presence of an active phosphohexose isomerase was demonstrated in wheat seedling extracts.

BIBLIOGRAPHY

1. Hassid, W. Z., and Putman, E. W., *Ann. Rev. Plant Physiol.*, **1,** 109 (1950).
2. Putman, E. W., and Hassid, W. Z., *J. Biol. Chem.*, **207,** 885 (1954).
3. Porter, H. K., in Williams, R. T., Biological transformations of starch and cellulose, Biochemical Society symposia, Cambridge, **11,** 27 (1953).
4. Vittorio, P. V., Krotkov, G., and Reed, G. B., *Canad. J. Bot.*, **32,** 369 (1954).
5. Greathouse, G. A., *Science*, **117,** 553 (1953).
6. Brown, S. A., and Neish, A. C., *Canad. J. Biochem. and Physiol.*, **32,** 170 (1954).
7. Putman, E. W., and Hassid, W. Z., *J. Biol. Chem.*, **196,** 749 (1952).
8. Hers, H. G., Edelman, J., and Ginsburg, V., *J. Am. Chem. Soc.*, **76,** 5160 (1954).
9. Isbell, H. S., Karabinos, J. V., Frush, H. L., Holt, N. B., Schwebel, A., and Galkowski, T. T., *J. Res. Nat. Bur. Standards*, **48,** 163 (1952).
10. Somogyi, M., *J. Biol. Chem.*, **117,** 771 (1937).
11. Nelson, N., *J. Biol. Chem.*, **153,** 375 (1944).
12. Bacon, J. S. D., and Bell, D. J., *Biochem. J.*, **42,** 397 (1948).
13. Roe, J. H., Epstein, J. H., and Goldstein, N. P., *J. Biol. Chem.*, **178,** 839 (1949).
14. Partridge, S. M., *Biochem. J.*, **42,** 238 (1948).
15. Bothner-By, A. A., and Gibbs, M., *J. Am. Chem. Soc.*, **72,** 4805 (1950).
16. Monier-Williams, G. W., *J. Chem. Soc.*, **119,** 803 (1921).
17. Gunsalus, I. C., and Gibbs, M., *J. Biol. Chem.*, **194,** 871 (1952).
18. Katz, J., Abraham, S., and Baker, N., *Anal. Chem.*, **26,** 1503 (1954).
19. Katz, J., Abraham, S., and Chaikoff, I. L., *Anal. Chem.*, **27,** 155 (1955).
20. Slein, M. W., *J. Biol. Chem.*, **186,** 753 (1950).
21. Racker, E., *Advances in Enzymol.*, **15,** 141 (1954).
22. Cook, M., and Lorber, V., *J. Biol. Chem.*, **199,** 1 (1952).
23. Dimant, E., Smith, V. R., and Lardy, H. A., *J. Biol. Chem.*, **201,** 85 (1953).
24. Minor, F. W., Greathouse, G. A., Shirk, H. G., Schwartz, A. M., and Harris, M., *J. Am. Chem. Soc.*, **76,** 1658 (1954).
25. Hassid, W. Z., Doudoroff, M., and Barker, H. A., *J. Am. Chem. Soc.*, **66,** 1416 (1944).
26. Leloir, L. F., and Cardini, C. E., *J. Am. Chem. Soc.*, **75,** 6084 (1953).

SYNTHESIS AND MICROBIOLOGICAL ACTIVITY OF SOME PANTOTHENIC ACID CONJUGATES

By GENE M. BROWN,* MIYOSHI IKAWA, and ESMOND E. SNELL

(From the Biochemical Institute and the Department of Chemistry, The University of Texas, and the Clayton Foundation for Research, Austin, Texas)

(Received for publication, October 15, 1954)

The postulated pathway of biosynthesis of coenzyme A is as follows (1, 2): pantothenic acid → pantothenylcysteine → pantetheine → 4′-phosphopantetheine → coenzyme A. Of these compounds, only pantothenic acid and pantetheine have been shown to support growth of lactic acid bacteria on a pantothenate-free medium (3, 4). Coenzyme A is inactive and the activities of pantothenylcysteine and phosphopantetheine have not been reported. However, *Acetobacter suboxydans* utilizes each of the above compounds that contains bound pantothenate much more effectively than free pantothenic acid (1, 5). Since each of these compounds is a substituted amide of pantothenic acid, it appeared possible that a combined carboxyl group in pantothenic acid might be the only prerequisite for enhanced activity. The synthesis of several such compounds and their activities for *A. suboxydans* and several lactic acid bacteria are reported here. In testing these compounds, the growth response of some organisms to pantothenic acid was found to vary considerably with the pH of the growth medium. These results also are described below.

Materials and Methods

For tests with *A. suboxydans* the pantothenate-free medium of Underkofler *et al.* (6) and the testing methods of Brown and Snell (1) were used. To obtain consistent assay results, it was found necessary to transfer the stock culture of this organism at weekly intervals. The medium and methods of Craig and Snell (3) were used for culture of *Lactobacillus acidophilus*, *Lactobacillus helveticus* 80, *Lactobacillus casei*, *Lactobacillus arabinosus* 17-5, and *Streptococcus faecalis* R. *Saccharomyces carlsbergensis* 4228 was cultured as described by Atkin *et al.* (7) for assay of pantothenic acid.[1]

For preparation of cell-free extracts, *A. suboxydans* was grown on a

* Present address, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

[1] Those organisms available from the American Type Culture Collection have the following numbers: *Acetobacter suboxydans* 621, *Lactobacillus acidophilus* 832, *Lactobacillus arabinosus* 8014, *Lactobacillus casei* 7469, *Streptococcus faecalis* 8043, and *Saccharomyces carlsbergensis* 9080.

larger scale in the same manner as for assays, but with an excess of pantothenic acid in the medium. After 48 hours, cells from 4 to 5 liters of medium were collected by centrifugation, washed once with water, and the 4 to 6 gm. of wet cells thus obtained suspended in 50 ml. of water, and disrupted by 15 minutes treatment in a Raytheon 10 kc. ultrasonic oscillator. The cell débris was removed by centrifugation at 0° and 13,000 $\times$ *g* for 45 minutes.

The barium salt of DL-4′-phosphopantetheine (8) was a gift from Dr. J. Baddiley. The preparation of pantethine (9), the mercuric mercaptide of pantetheine (9), and the diammonium salt of the mercuric mercaptide of pantothenylcysteine (1) have been described. Methyl pantothenate and the sodium salts of pantothenyl-β-alanine and pantothenyl-DL-alanine were supplied through the courtesy of Dr. E. L. Wittle of Parke, Davis and Company.

Synthesis of Some Pantothenic Acid Conjugates—All of the conjugates containing amino acids were synthesized from pantoyl lactone and the sodium salts of the appropriate β-alanyl peptides. All melting points are corrected.

N-Phthaloyl-β-alanylglycine—To a mixture of glycine (8 gm., 0.106 mole) and magnesium oxide (6.4 gm., 0.16 mole) in 300 ml. of water, chilled in an ice bath, were added 25.2 gm. (0.106 mole) of *N*-phthaloyl-β-alanyl chloride (10) in 100 ml. of dry dioxane over a period of 30 minutes with cooling and constant stirring (11). The mixture was stirred 1 hour longer at room temperature, then acidified with 35 ml. of 6 N hydrochloric acid. The copious white precipitate was filtered off and recrystallized from aqueous ethanol, yielding 20.9 gm. (71 per cent) of product melting at 188–194°, which was used without further purification in the next step.

A sample recrystallized twice more from aqueous ethanol melted at 212.5–213.5°.

$C_{13}H_{12}O_5N_2$.	Calculated.	C 56.5, H 4.4, N 10.1
	Found.	" 56.4, " 4.4, " 10.1

N-Phthaloyl-β-alanyl-L-leucine—L-Leucine (0.1 mole) was acylated with *N*-phthaloyl-β-alanyl chloride (0.08 mole) in the manner described above. The reaction mixture was acidified with 50 ml. of 6 N hydrochloric acid and placed in the cold for several hours. The solid was filtered off and the filtrate concentrated in a vacuum to about 100 ml. to yield additional solid. The combined crops were recrystallized from aqueous ethanol to give 18.8 gm. (71 per cent) of product melting at 127–130°.

A sample recrystallized twice more from aqueous ethanol melted at 131–133°. Turner (12) reports 113–117°. $[\alpha]_D^{24}$ −16° (2.3 per cent in ethanol).

$C_{17}H_{20}O_5N_2$. Calculated, N 8.4, found, N 8.3

β-Alanylglycine—A mixture of *N*-phthaloyl-β-alanylglycine (16.6 gm., 0.06 mole) in 180 ml. of ethanol and 60 ml. of 1 M hydrazine in ethanol was refluxed for 1 hour (11). The reaction mixture was concentrated under reduced pressure to a small volume, 100 ml. of water and 6 ml. of glacial acetic acid were added, and the mixture was warmed to 50° on a steam bath, cooled, and filtered. The filtrate was concentrated again to a sirup and the sirup redissolved in 100 ml. of water and reconcentrated. After two further reconcentrations to remove residual acetic acid, the final sirup was triturated with ethanol. The resulting solid was washed with ethanol and recrystallized from aqueous ethanol, yielding 4.7 gm. (53 per cent) of product melting at 229–230°. Hanson and Smith (13) report 226°.

β-Alanyl-L-leucine—β-Alanyl-L-leucine was prepared from *N*-phthaloyl-β-alanyl-L-leucine in the manner described by Sheehan and Frank (11) for the preparation of glycylglycine from its phthaloyl derivative. After recrystallization from aqueous ethanol, a 42 per cent yield of the free peptide, melting at 261–262° (decomposition), was obtained. Turner reports 259–260°. $[\alpha]_D^{34}$ −29° (2.5 per cent in water). Hanson and Smith (13) report $[\alpha]_D^{26}$ −31° (1.5 per cent in water); Nyman and Herbst (14) report $[\alpha]_D^{25}$ −27.4° (3.3 per cent in water).

Sodium Salts of D-*Pantothenylglycine,* D-*Pantothenyl-*L-*leucine, and* D-*Pantothenyl-*L-*histidine*—The sodium salts of the β-alanine peptides were prepared by dissolving these in the theoretical amount of 0.97 N sodium hydroxide and lyophilizing the resulting solutions. The dried products were dissolved in absolute ethanol (10 to 50 ml. per gm. of peptide), a 1.1 molar excess of D-pantolactone was added, and the mixtures were refluxed for 3 hours (method of Parke and Lawson (15) for the preparation of sodium pantothenate). The reaction mixtures were cooled, and the condensation products precipitated by addition of ether. The products were redissolved in a minimal amount of absolute ethanol, and again precipitated with ether. After washing with ether, the products were dried to crisp, white, hygroscopic solids in the vacuum desiccator.

This purification procedure removes all unchanged lactone, but unchanged peptide will appear in the final product. To assist in estimating purity, samples of the conjugates were hydrolyzed at 110° in 3 N hydrochloric acid for 48 hours and β-alanine was determined microbiologically with *S. carlsbergensis*. Under these conditions, calcium pantothenate gave the theoretical amount of β-alanine; the pantothenic acid conjugates gave high values for β-alanine in accordance with expectation. From these values and the nitrogen content, the purity of each product was estimated (Table I) and the calculation of activities corrected accordingly. The β-alanine peptides are without activity for all of the test organisms, and do not interfere with determinations of the activities of the conjugates.

Samples of pantothenyl-DL-alanine and of pantothenyl-β-alanine from Parke, Davis and Company were prepared by similar methods, and were estimated to have similar purities (private communication from Dr. E. L. Wittle).

D-*Pantothenamide*—Methyl D-pantothenate (4.75 gm.) was stirred with 100 ml. of concentrated aqueous ammonia at 0° for 6 hours, then allowed to stand at room temperature overnight. The reaction mixture was concentrated in a vacuum (under 50°) to dryness, and the sirup dissolved in 20 ml. of absolute ethanol and reconcentrated. After repeating the last step, the product was dissolved in 10 ml. of absolute ethanol, precipitated as a sirup by the addition of 50 ml. of ether, and washed with ether. The product was further purified by dissolving in 20 ml. of absolute ethanol and adding 60 ml. of ether. Under these conditions methyl pantothenate remains in solution. The resulting sirup was dried in a vacuum. Yield, 2.5 gm. (estimated purity, 80 per cent).

TABLE I

Analyses of Pantothenic Acid Conjugates

Sodium salt of	Analysis			Estimated purity, per cent
	N	Na	β-Alanine	
D-Pantothenylglycine, calculated	9.4	7.7	30	70
" found	11.8	7.4	35	
D-Pantothenyl-L-leucine, calculated	7.9	6.5	25	80
" found	8.8	6.8	37	
D-Pantothenyl-L-histidine, calculated	14.8	6.1	24	50
" found	18.6	5.2	33	

$C_9H_{18}O_4N_2$. Calculated. N 12.85, amide N 6.4, β-alanine 41
Found. " 13.7, " " 5.3, " 49

Results

Activities of Various Pantothenic Acid Conjugates for A. suboxydans—Pantetheine, pantothenylcysteine, and 4′-phosphopantetheine, as previously reported (1), are much more active than pantothenate in supporting growth of *A. suboxydans* (Group A, Table II). None of the other conjugates (Group B, Table II) approach in activity any of the compounds of Group A; however, pantothenamide and methyl pantothenate are considerably more active than pantothenate. The remaining compounds range in activity from pantothenylglycine, which is 65 per cent as active on a molar basis as pantothenate, to pantothenylleucine, which is about 10 per cent as active as pantothenate.

The results summarized in Table III show that cell-free extracts of *A. suboxydans* readily form free pantothenic acid from pantethine and from compounds of Group B that approach or surpass pantothenic acid in growth-promoting activity, but release no pantothenic acid (during the hydrolysis time employed) from the compounds that are less active in supporting growth. Aside from this qualitative correlation, the extent of hydrolysis by the extracts (methyl pantothenate = pantothenyl-β-alanine > pantethine = pantothenamide > pantothenylglycine) does not correlate with the growth-promoting activity (pantetheine > methyl pantothenate > pantothenamide > pantothenylglycine > pantothenyl-β-alanine).[2] Difference in ease of absorption of the various compounds undoubtedly is one factor that contributes to this lack of correlation. As shown subsequently, the high activities of pantothenamide and methyl pantothenate are due in part to their non-ionic nature and consequent ready absorption

TABLE II

Comparative Activities of Various Pantothenic Acid Conjugates in Supporting Growth of A. suboxydans

Compound	Amount required for half maximal growth, μmoles × 10^3 per 10 ml.
Calcium D-pantothenate	10.0
Group A	
D-Pantetheine (Hg mercaptide)	1.3
D-Pantothenylcysteine (Hg mercaptide)	2.2
DL-4′-Phosphopantetheine (Ba salt)	3.9*
Group B	
Methyl pantothenate	4.2
Pantothenamide	6.9
Pantothenylglycine†	15.4
Pantothenyl-β-alanine†	23.5
Pantothenyl-DL-alanine†	37.6
Pantothenyl-L-leucine†	96.0
Pantothenyl-L-histidine†	27.5

* The D isomer is twice as effective as the DL compound.
† As the sodium salts.

[2] It would not appear necessary that those compounds intermediate between pantothenic acid and coenzyme A be hydrolyzed prior to use by the organism. Although pantethine can be thus hydrolyzed, trials conducted so far indicate that pantothenylcysteine is not hydrolyzed.

by *A. suboxydans*. Thus the degree of activity of compounds of Group B probably depends both on the ease with which they are absorbed and the ease with which cellular enzymes convert them to free pantothenic acid.

Activities of Pantothenic Acid Conjugates for Lactic Acid Bacteria—Responses of four species of lactic acid bacteria to various pantothenic acid conjugates are shown in Table IV. One of these organisms, *L. helveticus* 80, utilizes pantetheine much more effectively than pantothenate (3); for the remaining three, pantothenate is somewhat more active than pantetheine. For all four species, compounds of Group A, which are known

TABLE III

Enzymatic Conversion of Pantothenic Acid Conjugates to Free Pantothenic Acid by Cell-Free Extracts of A. suboxydans

Each reaction mixture contained, per 3 ml. of volume, 0.05 μmole of conjugate to be tested, 1 ml. of 0.25 M tris(hydroxymethyl)aminomethane buffer (pH 7.5), and 1 ml. of cell-free extract of *A. suboxydans*. After incubation for 2 hours at 37°, free pantothenic acid was determined by microbiological assay with *Saccharomyces carlsbergensis* 4228, for which each of the conjugates is inactive. No free pantothenic acid was formed in the absence of the enzyme preparation.

Compound	Pantothenic acid formed, per cent of theoretical
Methyl pantothenate	86
Pantethine	72
Pantothenamide	71
Pantothenylglycine*	12
Pantothenyl-β-alanine*	85
Pantothenyl-DL-alanine*	0†
Pantothenyl-L-histidine*	0†
Pantothenyl-L-leucine*	0†

* As the sodium salt.

† Less than 2.4 per cent.

intermediates in the conversion of pantothenate to coenzyme A, are in general more active than compounds of Group B. Pantetheine was 5 to 6 times more active than phosphopantetheine for all organisms. This low activity of the latter compound recalls the inactivity of coenzyme A in promoting growth (3, 4) and suggests that the compound is not readily absorbed by the cells. Similarly, the relative inactivity of pantothenylcysteine suggests either that this compound does not readily pass into the cells or that, in contrast to *A. suboxydans* (16) and rats (2), these microorganisms utilize a mechanism for the conversion of pantothenate to coenzyme A in which pantothenylcysteine is not an intermediate.

For practical purposes, the compounds of Group B are inactive for these lactic acid bacteria; however, at sufficiently high levels, each of them per-

mits growth of each of the organisms. The activity of the compounds varies from organism to organism and therefore is not due to contaminating traces of pantothenic acid. Separate tests showed that none of the compounds inhibited response of these organisms or of *S. carlsbergensis* to limiting amounts of pantothenic acid.

TABLE IV

*Comparative Activities of Various Pantothenic Acid Conjugates in Supporting Growth of Lactic Acid Bacteria**

Compound	*L. helveticus*	*L. casei*	*L. arabinosus*	*S. faecalis*
	Amount required for half maximal growth, μmoles × 10^4 per 10 ml.			
Pantothenic acid (Ca salt)	420	1.3	1.1	0.7
Group A				
D-Pantothenylcysteine (Hg mercaptide)	5,700	66	620	17
D-Pantetheine (Hg mercaptide)	4.1	2.3	23	2.0
DL-4′-Phosphopantetheine (Ba salt)	24	12.6		10.2
Group B				
Pantothenamide	2,000	6.7	6.5	1.8
Pantothenylglycine*	>24,000	91	8.4	7.7
Pantothenyl-β-alanine†	>26,000	9	5,600	200
Pantothenyl-DL-alanine†	>26,000	620	>26,000	800
Pantothenyl-L-leucine†	>22,000	490	4,400	500
Pantothenyl-L-histidine†	>13,000	7000	>13,000	3800

* These activities were determined after 24 hour incubation periods and are subject to variations at shorter or longer incubation periods.

† As the sodium salts.

Effect of pH of Growth Medium on Response of Microorganisms to Pantothenic Acid and Its Conjugates—One possible explanation for the high activity of methyl pantothenate and pantothenamide as compared to pantothenic acid for *A. suboxydans* (Table II) is that the ionized carboxyl group of the latter compound decreases its absorbability, whereas the former nonionic compounds readily pass through the cell wall and are then converted to pantothenic acid (Table III). Absorption of many weak acids by certain plant cells is a function of the pH and increases as the amount of undissociated acid in the medium increases (17). If this were the case for *A. suboxydans*, progressively lowering the initial pH of the growth medium should cause pantothenic acid to become more and more active by depress-

ing its dissociation, but should have no effect on the activities of methyl pantothenate and pantothenamide. Results shown in Fig. 1 demonstrate that this is the case. The activity of pantothenic acid is increased 3-fold

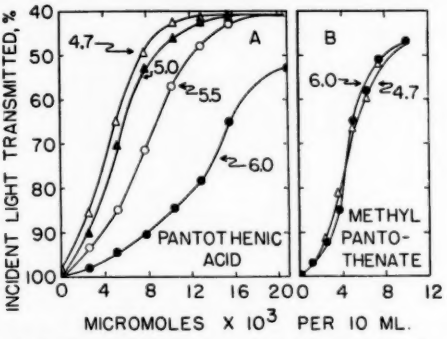


FIG. 1. The effect of pH on the requirement of *A. suboxydans* for pantothenic acid (*A*) or methyl pantothenate (*B*). The numbers adjacent to the curves show the initial pH of the growth medium.

TABLE V

Effect of Initial pH of Medium on Growth Response of A. suboxydans to Pantothenic Acid and Its Conjugates

Compound	Amount required for half maximal growth, μmoles × 10³ per 10 ml.			
	pH 6.0	pH 5.5	pH 5.0	pH 4.7
Calcium pantothenate	14.7	8.0	5.2	4.7
Undissociated pantothenic acid (calculated)	0.36	0.58	1.04	1.6
Methyl pantothenate	5.0	4.8	5.2	4.8
Pantothenamide	6.2	5.8	6.2	6.2
Pantetheine (Hg mercaptide)	1.6	2.0	2.6	2.7
Pantothenylcysteine (Hg mercaptide)	2.4	2.3	2.7	2.7
Pantothenylglycine (Na salt)	21.0	13.3	10.3	9.1

by lowering the initial pH of the medium from 6.0, the value normally used for the growth of *A. suboxydans*, to 4.7, while the activity of methyl pantothenate remains essentially unchanged (Fig. 1). Similar experiments have shown that lowering the pH of the medium also leaves the activity of pantothenamide unchanged (Table V). Initial pH values below 4.7 permit only scanty and inconsistent growth; hence their effects on utilization of pantothenic acid could not be tested.

The effect of pH on the response of *A. suboxydans* to various bound forms of pantothenate also was determined (Table V). The proportion of added calcium pantothenate present as undissociated pantothenic acid at each pH value was calculated from the ionization constant of pantothenic acid, 3.9×10^{-5} (18). If *A. suboxydans* absorbs only undissociated pantothenic acid, the amount of undissociated acid required to give a standard growth response should remain constant at each pH value. However, results (Table V) show that for a half maximal growth response this value is not constant, but decreases as the pH of the medium is increased. This indicates that *A. suboxydans* absorbs undissociated pantothenic acid principally but not exclusively. As expected from the fact that pantetheine

TABLE VI

*Effect of Initial pH of Medium on Growth Response of Lactic Acid Bacteria to Pantothenic Acid and Pantethine**

Organism	Pantothenic acid equivalents† required for half maximal growth, μmoles × 10^4 per 10 ml.					
	Calcium pantothenate			Pantethine		
	pH 6.5	pH 6.0	pH 5.5	pH 6.5	pH 6.0	pH 5.5
L. helveticus 80	520	280	150	3.6	3.5	3.0
L. acidophilus	350	290	260	3.6	3.9	3.2
L. casei	2.2	2.3	2.5	2.7	2.5	2.4
L. arabinosus 17-5	3.2	3.2	3.3	22	20	18

* The activities listed were determined after 22 hours incubation and vary to some extent with shorter or longer incubation periods.

† 1 mole of pantethine contains 2 equivalents of pantothenic acid.

contains no groups that ionize at these pH values, its activity is affected only slightly by the pH variations. Pantothenylglycine has a dissociable carboxyl group like that of pantothenic acid and shows similar variation in activity with changes in pH. However, at no pH is it as active as pantothenate. Pantothenylcysteine also possesses a carboxyl group, and might therefore be expected to be less active at higher pH values; this, however, did not prove to be the case. This compound contains an additional functional group (—SH) which apparently permits its absorption in spite of the presence of an ionized carboxyl group. The fact that the oxidized compound, pantothenylcystine, is essentially inactive lends some support to this idea.

The preference of *A. suboxydans* for undissociated pantothenic acid suggested that other bacteria also might utilize undissociated pantothenic acid more efficiently. Table VI shows the effect of pH on the response of

four lactic acid bacteria to pantothenate and to pantethine. The response of *L. helveticus*, and to a lesser extent of *L. acidophilus*, to pantothenate is markedly affected by pH. Although pantothenic acid is about 3 times more active for the former organism at pH 5.5 than at pH 6.5, pantethine is still about 50 times more active than pantothenate at the lower pH. Thus, the comparatively low activity of pantothenate for organisms of this type (many of which are known (3)) cannot be attributed to a failure to absorb dissociated pantothenic acid. The response of the four organisms to pantethine was unaffected by pH.

DISCUSSION

Pantetheine and pantothenylcysteine were reported (1, 5) to be 7 to 8 times more active on a molar basis than pantothenate in supporting growth of *A. suboxydans*. This difference was interpreted to mean either that the organism was deficient in its ability to condense pantothenate with cysteine to form pantothenylcysteine (1, 5) or that it did not absorb pantothenate from the medium as well as pantetheine or pantothenylcysteine (1). The results obtained above indicate that the low activity of pantothenate can, for the most part, be attributed to the latter cause. However, at all pH values tested pantetheine and pantothenylcysteine were more active than pantothenate. This may mean either that the ability of *A. suboxydans* to form pantothenylcysteine is somewhat impaired, though not to the extent originally considered probable, or that the sulfur-containing derivatives are absorbed more efficiently than undissociated pantothenic acid.

The ease with which pantothenylcysteine is absorbed from the medium by *A. suboxydans* suggests that the sulfhydryl (—SH) group present in this compound aids its passage through the cell wall and thus overcomes the deleterious effect of the ionized carboxyl group. Pantothenylcystine (the —SS— form of pantothenylcysteine) is essentially inactive for *A. suboxydans*, and both pantethine and 4′-phosphopantethine are several times more active in the reduced (—SH) than in the oxidized (—SS—) forms (1, 5). It was suggested (1) that the microorganism reduces the —SS— forms to the biologically active —SH compounds with difficulty. An alternative explanation suggested by the results discussed above is that only the —SH form of the compound is readily absorbed.

The rôle of 4′-phosphopantothenic acid in the intracellular metabolism of pantothenic acid is obscure. Small amounts of phosphorylated pantothenate have been detected in cell-free extracts of *A. suboxydans* and in the spent growth medium of *Neurospora* (unpublished). Furthermore, an enzyme present in cell-free extracts of *Proteus morganii* catalyzes phosphorylation of pantothenic acid by adenosinetriphosphate (19). Baddiley *et al.* (20) have reported a similar enzyme in extracts of *L. arabinosus*. These

results suggest that phosphopantothenic acid may play an important rôle in pantothenate metabolism; however, reports that *A. suboxydans* does not utilize phosphopantothenate for growth (21, 22, 5) have minimized its consideration as an intermediate. Phosphopantothenic acid contains three dissociable acid groups and no —SH group. In contrast to pantothenic acid, the compound would be fully ionized as a monovalent anion even at pH 4.7, the lowest pH at which *A. suboxydans* grows consistently. Its inactivity in supporting growth of *A. suboxydans* and of lactic acid bacteria may thus result wholly from its failure to pass the cell wall. To the extent that this is true, it cannot be excluded as a possible intermediate in the biosynthesis of coenzyme A. While the relative growth-promoting activities for *A. suboxydans* of pantothenic acid, pantothenylcysteine, pantetheine, 4′-phosphopantetheine, and coenzyme A increase in approximately the order listed and are thus consistent with biosynthetic relationships originally postulated on other grounds (1, 2, 16), this order of activity appears to result largely from permeability differences rather than from enzymatic deficiencies within the cell, and does not by itself provide convincing evidence in favor of one biosynthetic scheme for coenzyme A over another.

The danger of drawing quantitative conclusions from physiological responses to weak acids and bases without consideration of the effect that pH might have on these responses was pointed out by Simon and Beevers (17), and is aptly illustrated by these results with pantothenic acid in *A. suboxydans*.

SUMMARY

Synthesis of pantothenamide, pantothenylglycine, pantothenylleucine, and pantothenylhistidine is described. The growth-promoting activity of these compounds for several organisms is compared with that of several other derivatives of pantothenic acid.

Of these compounds, only pantothenic acid promotes growth of *Saccharomyces carlsbergensis*. For *Acetobacter suboxydans* their activity follows the order 4′-phosphopantetheine $\geqq$ pantetheine = pantothenylcysteine > methyl pantothenate $\cong$ pantothenamide > pantothenic acid > pantothenylglycine > pantothenyl-β-alanine > pantothenyl-L-histidine > pantothenyl-DL-alanine > pantothenyl-L-leucine.

Only pantothenic acid, pantetheine, and 4′-phosphopantetheine have high activity for the lactic acid bacteria tested. Either pantothenic acid or pantetheine may far surpass the other in activity, depending upon the organism (3, 4). 4′-Phosphopantetheine is less than 20 per cent as active as pantetheine in all cases. Pantothenamide, pantothenylglycine, and pantothenyl-β-alanine possess significant activity (up to 40 per cent that

of pantothenic acid) for some, but not all, of these organisms; the other compounds support growth only at extremely high levels. None of the compounds acted as antagonists of pantothenic acid or of pantetheine for any of the test organisms.

The activity of calcium pantothenate but not that of methyl pantothenate, pantothenamide, or pantetheine for *A. suboxydans* increased markedly as the pH of the growth medium was decreased. Apparently undissociated pantothenic acid is more easily absorbed than its anion. The activity of pantothenylglycine varies with pH like that of pantothenate; that of pantothenylcysteine does not. It is concluded that presence of an —SH group permits efficient absorption despite the presence of the ionized carboxyl group. For the lactic acid bacteria tested, a similar pH effect was noted with *Lactobacillus helveticus* and *Lactobacillus acidophilus;* under no conditions did the activity of pantothenic acid approach that of pantethine for these organisms. The response to pantothenic acid of the several lactic acid bacteria used for routine microbiological determinations of this substance (*Lactobacillus arabinosus*, *Lactobacillus casei*) was not affected by variations in pH.

Cell-free extracts of *A. suboxydans* contain one or more enzymes that rapidly hydrolyze methyl pantothenate, pantothenamide, pantethine, pantothenyl-β-alanine, and pantothenylglycine to yield free pantothenic acid. The growth-promoting activity for this organism of conjugates of pantothenic acid is correlated qualitatively but not quantitatively with the ability of such extracts to hydrolyze them.

The significance of certain of these findings is discussed briefly.

Addendum—Since completion of this work, a paper by J. Baddiley and A. P. Mathias (23) has appeared in which the synthesis and activity of several of the pantothenic acid peptides tested here are also described. So far as their microbiological results are concerned, we are in perfect agreement.

BIBLIOGRAPHY

1. Brown, G. M., and Snell, E. E., *J. Bact.*, **67,** 465 (1954).
2. Hoagland, M. B., and Novelli, G. D., *J. Biol. Chem.*, **207,** 767 (1954).
3. Craig, J. A., and Snell, E. E., *J. Bact.*, **61,** 283 (1951).
4. Snell, E. E., and Brown, G. M., *Advances in Enzymol.*, **14,** 49 (1953).
5. King, T. E., and Cheldelin, V. H., *Proc. Soc. Exp. Biol. and Med.*, **84,** 591 (1953).
6. Underkofler, L. A., Bantz, A. C., and Peterson, W. H., *J. Bact.*, **45,** 183 (1943).
7. Atkin, L., Williams, W. L., Schultz, A. S., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **16,** 67 (1944).
8. Baddiley, J., and Thain, E. M., *J. Chem. Soc.*, 1610 (1953).
9. Wittle, E. L., Moore, J. A., Stipek, R. W., Peterson, F. E., McGlohon, V. M., Bird, O. D., Brown, G. M., and Snell, E. E., *J. Am. Chem. Soc.*, **75,** 1694 (1953).
10. Walton, E., Wilson, A. N., Holly, F. W., and Folkers, K., *J. Am. Chem. Soc.*, **76,** 1146 (1954).

XUM

11. Sheehan, J. C., and Frank, V. S., *J. Am. Chem. Soc.*, **71,** 1856 (1949).
12. Turner, R. A., *J. Am. Chem. Soc.*, **75,** 2388 (1953).
13. Hanson, H. T., and Smith, E. L., *J. Biol. Chem.*, **175,** 833 (1948).
14. Nyman, M. A., and Herbst, R. M., *J. Org. Chem.*, **15,** 108 (1950).
15. Parke, H. C., and Lawson, E. J., *J. Am. Chem. Soc.*, **63,** 2869 (1941).
16. Brown, G. M., and Snell, E. E., *J. Am. Chem. Soc.*, **75,** 2782 (1953).
17. Simon, E. W., and Beevers, H., *Science*, **114,** 124 (1951).
18. Williams, R. J., *Advances in Enzymol.*, **3,** 253 (1943).
19. Ward, G. B., Brown, G. M., and Snell, E. E., *J. Biol. Chem.*, **213,** 869 (1955).
20. Baddiley, J., Hughes, D. E., Mathias, A. P., and Pierpoint, W. S., *Biochem. J.*, **56,** p. xxii (1954).
21. King, T. E., and Strong, F. M., *Science*, **112,** 562 (1950).
22. Baddiley, J., and Thain, E. M., *J. Chem. Soc.*, 246 (1951).
23. Baddiley, J., and Mathias, A. P., *J. Chem. Soc.*, 2803 (1954).

PHOSPHORYLATION OF PANTOTHENIC ACID AND PANTETHINE BY AN ENZYME FROM PROTEUS MORGANII

By G. B. WARD, GENE M. BROWN,* AND ESMOND E. SNELL

(From the Biochemical Institute and the Department of Chemistry, The University of Texas, and the Clayton Foundation for Research, Austin, Texas)

(Received for publication, October 15, 1954)

Before the nature of "bound" pantothenic acid was established, McIlwain and Hughes (1) showed that incubation of pantothenic acid with various streptococci or *Proteus morganii* led to its "inactivation" as a growth factor for these organisms. This inactivation reaction was dependent upon glycolysis, but independent of growth and the presence of oxygen, and was inhibited by pantoyltaurine (2). McIlwain (3) assumed that both glycolysis and pantothenic acid are necessary for formation of some substance necessary for growth.

It now seems probable that this inactivation reaction represented one or more reactions associated with conversion of pantothenic acid to coenzyme A. The latter product and each of the proposed intermediates between it and pantothenic acid are inactive as growth factors for yeast and many bacteria (4–6).

In a previous communication (7) it was stated that one such inactivation reaction in *P. morganii* depended upon the presence of adenosinetriphosphate (ATP) and cysteine, yielding a product identical in behavior to pantothenylcysteine. The latter product, though inactive for most bacteria (5–7), is highly active for *Acetobacter suboxydans* (5), and appears to be an intermediate in coenzyme A synthesis (5, 7, 8). During this work, acetone-dried cells of *P. morganii* also were observed to inactivate pantothenic acid as a growth factor for yeast in the absence of cysteine, provided ATP were present. Phosphatase treatment regenerated pantothenic acid inactivated by the cysteine-independent reaction, but not that inactivated by the cysteine-dependent reaction, thus permitting their ready differentiation.

The enzyme associated with this cysteine-independent inactivation of pantothenic acid has been obtained in cell-free extracts. Its partial purification and some of its properties are described below.

* Present address, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

XUM

EXPERIMENTAL

Assay Procedures—Pantothenic acid was determined with *Saccharomyces carlsbergensis* 4228 (9), for which coenzyme A (4), pantethine (4), pantothenylcysteine (6, 7), and each of the phosphorylated forms of pantothenic acid (10, 11) are inactive. Pantethine was determined with *Lactobacillus helveticus* 80 (4); pantothenic acid, pantothenylcystine, and coenzyme A are essentially inactive for this organism, and 4′-phosphopantetheine is less than 20 per cent as active as pantethine (4, 6).

Source of Enzyme—P. morganii (A. T. C. C. No. 8019) was carried on nutrient agar slants with monthly transfer. Inocula were prepared by suspending growth from a 24 hour-old culture on such a slant in 5 ml. of sterile water; 2.5 ml. of the resulting suspension were used to inoculate each 500 ml. of basal medium. The basal medium was that described by Pelczar and Porter (12) with the alkali-treated peptone, nicotinic acid, and cystine replaced by 0.5 per cent yeast extract. 6 liter lots of this medium in 9 liter Pyrex jars were sterilized, cooled, inoculated, and incubated with aeration at 30° for 24 hours. Cells were harvested in a Sharples centrifuge and washed twice with water.

Enzyme Assay—Activity of the enzyme is measured as the difference between the pantothenic acid initially present and that remaining after an appropriate time interval. To avoid inaccuracies attendant upon this value being a small difference between two large numbers, the reaction must go substantially toward completion. For this reason, the amount of pantothenic acid added as substrate was suboptimal, and ATP was used in excess. After appropriate preliminary experiments, a total reaction volume of 4 ml. containing 62.5 γ (0.292 μmole) of calcium pantothenate, 13 mg. (22 μmoles) of ATP, 160 γ of $MgSO_4 \cdot 7H_2O$, 0.25 μmole of tris-(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, and enzyme was adopted. 1 unit of enzyme activity was defined as that amount of preparation that would catalyze inactivation of 1 γ of calcium pantothenate in 90 minutes under these conditions. Variations from these conditions are specified with Tables I and II. Protein was determined turbidimetrically by the procedure of Stadtman *et al.* (13).

Preparation of Enzyme—Active preparations of the enzyme are obtainable from acetone- or vacuum-dried cells, but are less active than those obtained by sonic disruption of the freshly harvested cells (Table I). For the latter purpose, washed cells from 5 liters of medium were suspended in 50 ml. of 0.2 M phosphate buffer, pH 7.0, and subjected to 10 kc. vibrations in a Raytheon ultrasonic oscillator for 25 minutes. Cell débris was removed by high speed centrifugation in the cold. The combined extracts (55,000 units, 1.1 units per mg. of protein) from cells from 36 liters of medium were refrigerated at 4° for several days, and precipitated, inactive

proteins were removed by centrifugation. Further inactive protein precipitated on standing for 6 hours at 37° and was discarded. A small amount of additional inactive material was precipitated by treatment with protamine sulfate, as described for removal of nucleic acids (15). The supernatant solution from the latter precipitation (34,100 units, 7.2 units per mg. of protein), pH 6.9, was treated at room temperature with an equal volume of saturated ammonium sulfate solution, and the precipitate was collected by centrifugation and dissolved in one-fourth the original volume of water. It was then dialyzed overnight against two changes of distilled

TABLE I

Comparative Activities of Various Enzyme Preparations in Inactivation of Pantothenic Acid

Preparation*	Pantothenic acid inactivated
	μmoles × 10^3
1. Acetone-dried cells†	3.4
2. Aqueous extract of (1)‡	1.2
3. Vacuum-dried cells§	4.2
4. Aqueous extract of (3)‡	1.5
5. Alumina-ground cells‖	2.9
6. Sonically disrupted	6.7

* 40 mg. of cells, or extract prepared from them, were used in the assay procedure described in the text.

† Washed cells poured into 10 volumes of cold acetone (2°), stirred 15 minutes, filtered, washed with 10 volumes of ether, and dried over P_2O_5 in a vacuum.

‡ 40 mg. of dried cells extracted overnight at 4° with 1 ml. of 0.25 M Tris buffer, pH 7.0; cells removed by centrifugation.

§ Wet cells were dried in a vacuum over KOH for 24 hours.

‖ Method of McIlwain (14).

water. The final preparation (26,600 units, 23.9 units per mg. of protein) represented 48 per cent of the initial activity, purified approximately 22-fold over the initial extract.

Nature and Substrate Specificity of Inactivation Reaction—Under the assay conditions used here, a tremendous excess of ATP is required for the inactivation reaction (Fig. 1). This is due in part to the low concentration of pantothenate used (quantitative conversion to the product was achieved) and in part to the presence of an active ATPase in the enzyme preparation used. The specificity of the reaction with respect to ATP has not been investigated, however, and the possible participation of other nucleotide polyphosphates (16, 17), possibly present as a contaminant of the ATP used, is not excluded. That the reaction is a phosphorylation of pantothenic acid is shown by the quantitative recovery of pantothenic acid fol-

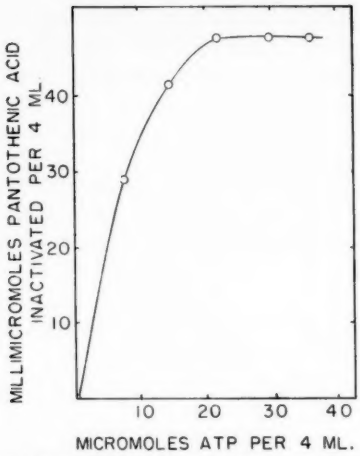


FIG. 1. The relationship of ATP concentration to the phosphorylation of pantothenic acid. The incubation mixture contained 0.048 μmole of calcium pantothenate (12.5 γ) and 46 units of kinase per 4 ml., and was incubated 3 hours at 37°. Other conditions are as in the text.

TABLE II

Phosphorylation of Pantothenic Acid, Pantetheine, and Pantethine by Kinase and Hydrolysis of Products by Intestinal Phosphatase

The values are given in micromoles × 10^3 per 4 ml.

Trial	Substrate	Substrate present in reaction mixture		
		Before reaction	After reaction	After reaction and intestinal phosphatase treatment*
1†	Pantothenic acid	37	27.5	41
	" "	260	82	250
	" "	260	87	263
2‡	" "	34	25.5	39
	Mercury mercaptide of pantetheine§	17	11	13
	Pantethine§	17	10.3	16
3‖	Pantothenic acid	34	30	
	Pantethine	34	29.5	
	Mercury mercaptide of pantetheine	34	29.8	

* 10 mg. of Armour's purified intestinal phosphatase were added to the reaction mixture which was incubated an additional 90 minutes at 37° before assay.

† 13 units of kinase per 4 ml.; incubated 90 minutes at 37°.

‡ 13 units of kinase per 4 ml.; incubated 90 minutes at 37°.

§ These products contain 2 equivalents of pantothenic acid per mole.

‖ The enzyme concentration was reduced to 0.3 unit per 4 ml.

lowing treatment with Armour's purified intestinal phosphatase (Table II). The product of the enzymatic phosphorylation was identified as 4′-phosphopantothenic acid with the aid of paper chromatography. Appropriate amounts of the enzymatic reaction mixture and control substances were spotted on separate strips of filter paper. These were developed overnight with *n*-propanol-ammonium hydroxide (33 per cent ammonia)-water (60:30:10 parts by volume), then dried, sprayed with a solution of Armour's

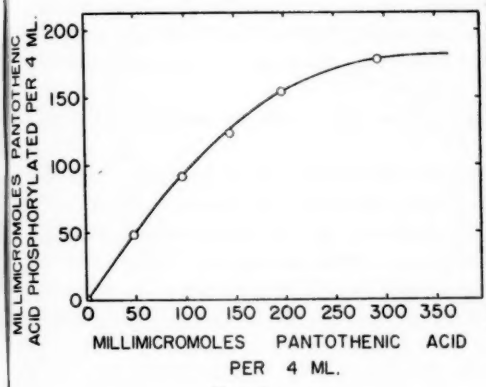


FIG. 2

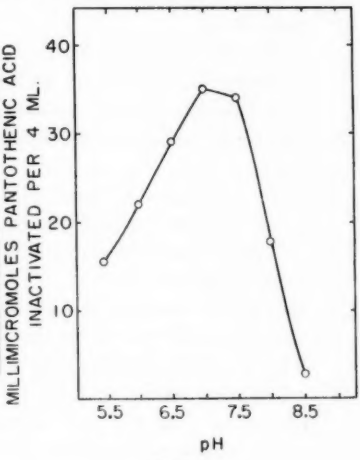


FIG. 3

FIG. 2. The effect of substrate concentration on the phosphorylation of pantothenic acid. 46 units of kinase were used per 4 ml.; other conditions are as in the text.

FIG. 3. Effect of pH on the enzymatic phosphorylation of pantothenic acid. 0.048 μmole of pantothenic acid and 10 units of kinase per 4 ml. Phosphate buffers (0.2 M) from pH 5.5 to 6.5; Tris buffer (0.25 M) for pH 7.0 to pH 8.5; other conditions are as in the text.

intestinal phosphatase (4 mg. per ml. of 0.1 M Tris buffer, pH 7.5), and incubated in a closed container at 37° for 90 minutes. Zones were located by the bioautograph technique with *S. carlsbergensis* as the test organism. The enzymatic inactivation product (R_F = 0.50) migrated identically with a sample of 4′-phosphopantothenic acid kindly supplied by Dr. J. Baddiley.

From Table II it is apparent that the same enzyme preparation phosphorylates both pantethine and pantetheine at approximately the same rate as pantothenic acid. The amount of pantothenic acid phosphorylated was decreased by simultaneous addition of pantethine; *i.e.*, the two products appear to be competitive substrates for the enzyme. In a quanti-

tative experiment of this nature, 0.1 μmole of calcium pantothenate and 600 units (≃ 0.1 μmole of pantetheine) of pantethine were incubated with 43 units of enzyme for 90 minutes. During this time, 50 per cent of the pantothenic acid and 86 per cent of the pantethine were inactivated by phosphorylation. In the absence of pantethine, the pantothenic acid was completely phosphorylated. From this result, pantethine would appear to be somewhat the preferred substrate. By analogy with the product formed from pantothenic acid, that from pantethine is assumed to be 4′-phosphopantethine.

The Michaelis constant for the kinase with pantothenic acid as substrate is approximately 2.5×10^{-5} M (Fig. 2). It will be observed from Fig. 2 that the substrate concentration used for assay of the kinase, for reasons discussed earlier, is considerably below that necessary to saturate the enzyme.

Miscellaneous Properties—The kinase has a sharply defined pH optimum between 7.0 and 7.5 (Fig. 3).

A requirement for magnesium ion was demonstrable following dialysis of the purified enzyme. With 50 γ of calcium pantothenate and 55 units of dialyzed enzyme in the reaction mixture, 12 γ of calcium pantothenate were phosphorylated in the absence of added magnesium, whereas 31 γ were phosphorylated by the magnesium-supplemented enzyme.

Limited studies of heat stability showed no inactivation after 90 minutes at 37°, slightly over 75 per cent inactivation after 30 minutes at 50°, and complete destruction of enzymatic activity in 30 minutes at 60°.

DISCUSSION

The kinase described here resembles the pantetheine kinase from pigeon liver described by Levintow and Novelli (18) in pH optimum, in its Michaelis constant for its substrates, and in the high requirement for ATP relative to pantetheine required for maximal activity. The *Proteus* enzyme appears to be considerably more stable to heat than the enzyme from pigeon liver. The substrate specificity of the pigeon liver enzyme was not determined; whether it is specifically a pantetheine kinase or, like the enzyme from *Proteus* studied here, has a more general specificity, is not known. The presence of an apparently similar enzyme in *Lactobacillus arabinosus* has been noted without details by Baddiley *et al.* (19).

Phosphorylation of pantothenic acid by the enzyme raises the question of the possible importance of phosphopantothenate as an intermediate in pantothenate metabolism. Treatment of several natural products, and especially the spent growth medium of *Neurospora crassa*, with purified intestinal phosphatase results in enhanced pantothenic acid activity for *S. carlsbergensis*. Of the known bound forms of pantothenic acid, only phos-

XUM

phopantothenic acid is converted to a growth factor for yeast by this treatment. This compound might be visualized as an intermediate in coenzyme A synthesis by Route A.

(*A*) Pantothenate → phosphopantothenate → phosphopantothenylcysteine → phosphopantetheine → dephosphocoenzyme A → coenzyme A

which might be an alternative to Route B, all reactions of which have been shown to occur in cell-free extracts (7, 8, 18):

(*B*) Pantothenate → pantothenylcysteine → pantetheine → phosphopantetheine → dephosphocoenzyme A → coenzyme A

As noted elsewhere (6), permeability relationships are such that the participation of phosphopantothenic acid in a scheme such as Route A (which might occur together with or as an alternative to Route B) cannot be discounted because of its inactivity in supporting growth. It is also possible, however, that phosphopantothenate formation by the kinase described here represents a metabolic *cul-de-sac*, and that the essential function of the enzyme is as a pantetheine kinase in accord with biosynthetic Route B. Conversion of pantothenate by *P. morganii* to a product identical in growth-promoting characteristics with pantothenylcysteine has been noted previously (7). It appears beyond question that one or both of these two reactions are inhibited by pantoyltaurine, as observed originally by McIlwain (2).

SUMMARY

Preparation and partial purification of a kinase from *Proteus morganii* that phosphorylates pantothenic acid, pantethine, and pantetheine with approximately equal facility are described. The Michaelis constant for pantothenic acid is approximately 2.5×10^{-5} M; adenosinetriphosphate and magnesium ions are required for the reaction. The pH optimum lies between 7.0 and 7.5. The product formed from pantothenic acid was identified chromatographically as 4′-phosphopantothenic acid. The enzyme resembles in many respects the pantetheine kinase from pigeon liver (18), but is more stable to heat and is less specific with respect to substrate. The possible significance of phosphopantothenic acid as a metabolic intermediate is discussed briefly.

BIBLIOGRAPHY

1. McIlwain, H., and Hughes, D. E., *Biochem. J.*, **38**, 187 (1944); **39**, 133 (1945).
2. McIlwain, H., *Biochem. J.*, **39**, 279 (1945); **40**, 269 (1946).
3. McIlwain, H., *Nature*, **158**, 898 (1946).
4. Craig, J. A., and Snell, E. E., *J. Bact.*, **61**, 283 (1951).
5. Brown, G. M., and Snell, E. E., *J. Bact.*, **67**, 465 (1954).

6. Brown, G. M., Ikawa, M., and Snell, E. E., *J. Biol. Chem.*, **213,** 855 (1955).
7. Brown, G. M., and Snell, E. E., *J. Am. Chem. Soc.*, **75,** 2782 (1953).
8. Hoagland, M. B., and Novelli, G. D., *J. Biol. Chem.*, **207,** 767 (1954).
9. Atkin, L., Williams, W. L., Schultz, A. S., and Frey, C. N., *Ind. and Eng. Chem., Anal. Ed.*, **16,** 67 (1944).
10. King, T. E., and Strong, F. M., *J. Biol. Chem.*, **189,** 315 (1951).
11. King, T. E., and Strong, F. M., *Science*, **112,** 562 (1950).
12. Pelczar, M. J., Jr., and Porter, J. R., *J. Biol. Chem.*, **139,** 111 (1941).
13. Stadtman, E. R., Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **191,** 365 (1951).
14. McIlwain, H., *J. Gen. Microbiol.*, **2,** 288 (1948).
15. Korkes, S., del Campillo, A., and Ochoa, S., *J. Biol. Chem.*, **187,** 891 (1950).
16. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209,** 23 (1954).
17. Ayengar, P., Gibson, D. M., and Sanadi, D. K., *Biochim. et biophys. acta*, **13,** 309 (1954).
18. Levintow, L., and Novelli, G. D., *J. Biol. Chem.*, **207,** 761 (1954).
19. Baddiley, J., Hughes, D. E., Mathias, A. P., and Pierpoint, W. S., *Biochem. J.*, **56,** p. xxii (1954).

XUM

THE ISOLATION OF α,α'-DIAMINOPIMELIC ACID FROM LACTOBACILLUS ARABINOSUS

By MIYOSHI IKAWA and JEAN S. O'BARR

(From the Biochemical Institute, The University of Texas, and the Clayton Foundation for Research, Austin, Texas)

(Received for publication, October 18, 1954)

In 1950 Work reported the isolation of a new naturally occurring amino acid from the water-insoluble portion of *Corynebacterium diphtheriae* cells (1) and showed it to be α,α'-diaminopimelic acid (DAP) (2, 3). The substance has subsequently been isolated from the cells of *Mycobacterium tuberculosis* (3) and from the culture filtrates of an *Escherichia coli* mutant (4, 5). A survey of the distribution of DAP in various microorganisms was recently made by Work and Dewey (6) by paper chromatographic techniques. These authors reported the presence of DAP in *Lactobacillus arabinosus* (plantarum) in amounts greater than 0.8 per cent of the dry weight of the cells.

During the course of investigations on the constituents of the cells of *L. arabinosus*, appreciable amounts of what appeared to be DAP were detected chromatographically in hydrolysates of the cell protein fractions. Its presence has been confirmed by isolation and shown to be DAP by elementary analysis, x-ray comparison with a sample obtained from another source (Table I), and paper chromatographic comparisons with a synthetic sample of DAP (kindly furnished by Dr. H. K. Mitchell of the California Institute of Technology). Paper chromatographic comparisons with synthetic α,α'-diaminoadipic acid (also supplied by Dr. Mitchell) served to distinguish it from the latter compound.

The isolation of DAP in amounts of approximately 1 per cent of the dry weight of the cells in a fraction derived principally from protein suggests that it is a constituent of the proteins of *L. arabinosus* and not a substance performing a catalytic rôle. Such conclusions have been reached by Work (2, 3) with respect to the DAP of *C. diphtheriae* and by Gendre and Lederer (7) in the case of the *Mycobacteria*.

The absence of any detectable rotation indicates that the DAP from *L. arabinosus* has the meso configuration similar to that from the cells of *C. diphtheriae* (3) and *E. coli* culture filtrates (5).

EXPERIMENTAL[1]

Fractionation of Cells—Lyophilized *L. arabinosus* 17-5 cells (10 gm.) were extracted with 200 ml. of water on a steam bath for ½ hour and the mixture was cooled and centrifuged to yield a clear supernatant solution (Fraction A) and an insoluble residue. The residue was suspended in 180 ml. of water at 90°, 20 ml. of trichloroacetic acid (1 gm. per ml. in water) were added, and the mixture was heated for 15 minutes to remove nucleic acids (8), cooled rapidly, and centrifuged to yield a supernatant fluid (Fraction B) and an insoluble residue, which was washed with 100 ml. of water. The residue was refluxed with 200 ml. of 1 N hydrochloric acid for 15 minutes, cooled rapidly, and centrifuged to yield a supernatant solution

TABLE I

*Comparison of Powder X-ray Diffraction Patterns of DAP from L. arabinosus and E. coli**

DAP from *L. arabinosus*		DAP from *E. coli*†	
d_{hkl}	Relative intensity	d_{hkl}	Relative intensity
A		*A*	
14.2	<0.1	14.1	<0.1
8.51	0.1	8.53	0.2
7.09	<0.1	7.07	<0.1
6.21	<0.1	6.23	<0.1
5.36	0.3	5.35	0.5
4.61	0.5	4.66	0.7
4.26	0.5	4.27	1.0
3.87	0.3	3.90	0.4
		3.74	1.0
3.63	1.0	3.66	1.0
		3.53	0.4
3.33	0.3	3.33	0.4
2.85	<0.1	2.85	0.1
2.68	0.3	2.68	0.5
2.58	0.2	2.59	0.3
2.40	0.1	2.39	0.3
2.29	0.2	2.28	0.2
2.16	<0.1	2.18	0.1
1.71	<0.1	1.68	<0.1

* The x-ray diffractions were kindly run by Dr. E. J. Weiss of the Department of Ceramic Engineering (Cu K_α wave-length).

† Kindly supplied by Dr. L. D. Wright of Sharp and Dohme, Inc., and isolated from an *E. coli* culture medium (5).

[1] Microanalyses by the Elek Micro Analytical Laboratories, Los Angeles, California.

(Fraction C) and a residue (Fraction D). Fraction C, on concentrating *in vacuo* to a sirup and further drying in a vacuum desiccator, yielded about 4 gm. of solid. After hydrolysis, in only Fraction C, which is derived principally from the insoluble cell proteins, could DAP be detected chromatographically.

Isolation of DAP—Four 1 gm. samples of Fraction C were sealed in tubes with 25 ml. portions of 3 N hydrochloric acid and heated at 110–120° for 18 hours. The hydrolysates were combined, concentrated to dryness *in vacuo*, redissolved in water, and the solution filtered and chromatographed on a Dowex 50 column (22 × 450 mm.) in the acid form (9). The column was developed with increasing concentrations of hydrochloric acid (9), and the DAP in the effluent located by quantitative ninhydrin determinations on the effluent fractions and by paper chromatography. The DAP coemerges with leucine or isoleucine. The DAP-containing fractions were concentrated to dryness *in vacuo*, and the residue triturated and washed with absolute ethanol. The ethanol-insoluble solid was dissolved in about 1 ml. of water, centrifuged, and 3 volumes of absolute ethanol added to the supernatant fluid. A white crystalline solid was obtained, which was centrifuged, washed with absolute ethanol, then ether, and dried. Yield, 96 mg. (about 1 per cent of the dry weight of the cells). On paper chromatography the sample showed only a single spot and the absence of leucine or isoleucine.

Analysis of a sample dried over P_2O_5 *in vacuo* for several days indicated that it is a partial hydrochloride. No optical rotation could be detected in the sample. $[\alpha]_D^{33} < \pm 1°$ (C = 1.15 per cent in 2 N HCl).

$C_7H_{14}O_4N_2 \cdot 0.3HCl$.	Calculated.	C 41.8, H 7.2, N 13.9
	Found.	" 42.0, " 7.1, " 13.9

The free amino acid was prepared by dissolving the hydrochloride in 30 ml. of water, passing the solution through a column containing 20 gm. of IR-4B resin in the OH^- form, and developing the column with water. The ninhydrin-positive fractions of the effluent were concentrated *in vacuo* to about 6 ml., the concentrate warmed to dissolve the solid which had crystallized, and 18 ml. of absolute ethanol were added. After standing in the cold overnight, the solid was centrifuged, washed with absolute ethanol, then ether, and dried *in vacuo* over P_2O_5. Yield, 68 mg. from 96 mg. of the hydrochloride.

$C_7H_{14}O_4N_2$.	Calculated.	C 44.2, H 7.4, N 14.7
	Found.	" 43.6, " 7.6, " 13.7

SUMMARY

The isolation of α,α'-diaminopimelic acid from cells of *Lactobacillus arabinosus* 17-5 is described. The isolated amino acid accounts for approxi-

mately 1 per cent of the dry weight of the cells. The amino acid is combined chemically in a trichloroacetic acid-insoluble (protein) fraction of the cell and appears to be optically inactive.

BIBLIOGRAPHY

1. Work, E., *Biochim. et biophys. acta*, **5**, 204 (1950).
2. Work, E., *Nature*, **165**, 74 (1950).
3. Work, E., *Biochem. J.*, **49**, 17 (1951).
4. Work, E., and Denman, R., *Biochim. et biophys. acta*, **10**, 183 (1953).
5. Wright, L. D., and Cresson, E. L., *Proc. Soc. Exp. Biol. and Med.*, **82**, 354 (1953).
6. Work, E., and Dewey, D. L., *J. Gen. Microbiol.*, **9**, 394 (1953).
7. Gendre, T., and Lederer, E., *Biochim. et biophys. acta*, **8**, 49 (1952).
8. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
9. Stein, W. H., and Moore, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 179 (1950).

THE ACTIVATION OF HUMAN PLASMINOGEN BY STREPTOKINASE*

By WALTER TROLL† and SOL SHERRY‡

(From the May Institute for Medical Research of the Jewish Hospital Association, Cincinnati, Ohio)

(Received for publication, August 30, 1954)

Human plasma contains a proteolytic enzyme precursor termed plasminogen (1) or profibrinolysin (2), which, upon activation by physical agents or specific bacterial kinases, is converted to plasmin (1) or fibrinolysin (2). Streptokinase (henceforth referred to as SK), an extracellular hemolytic streptococcal product, has been a commonly used activator for human plasminogen. Two mechanisms have been proposed for the activation of plasminogen by SK: (*a*) plasminogen is converted to plasmin, a new substance, by enzymatic activation (3) and (*b*) the conversion of plasminogen to plasmin results from a "stoichiometric" interaction of SK with plasminogen (4, 5).

Studies with synthetic substrates on the activation of plasminogen by SK reveal that human plasminogen preparations contain two factors: a *proactivator*, converted by SK in stoichiometric fashion to a plasminogen activator, and *plasminogen*, which is enzymatically converted by the activator to the proteolytic enzyme plasmin. Evidence for a two-step activation of plasminogen by SK, in agreement with that recently suggested by others (6, 7), was obtained through three types of studies: (*a*) fractionation experiments demonstrating two factors in SK-activated plasminogen preparations, *i.e.* a lysine esterase alone, and a proteolytic enzyme capable of splitting casein, arginine, and lysine esters; (*b*) inhibition experiments relating the lysine esterase to the plasminogen activator; and (*c*) activation experiments demonstrating the reversible stoichiometric activation of the lysine esterase by SK and the enzymatic activation of the proteolytic enzyme.

* Supported by grants from the National Institutes of Health, United States Public Health Service, and the Lederle Laboratories Division, American Cyanamid Company.

† Present address, Cancer Research Institute of the New England Deaconess Hospital, Boston, Massachusetts.

‡ Present address, Department of Medicine, The Jewish Hospital of St. Louis, St. Louis, Missouri.

Preparations and Methods

Plasminogen preparations were prepared according to the method of Kline (8) from human plasma Fraction III.[1] A commercial preparation of SK[2] was used. Lysine ethyl ester (LEe), tosylarginine methyl ester (TAMe), and tosylarginine butyl ester (TABe) were used as substrates and prepared as described (9).

Fibrinolytic activity, with a bovine fibrinogen substrate, casein proteolysis, and TAMe splitting were measured by reported methods (9).[3]

A photometric assay for TABe hydrolysis, which could be employed in the presence of LEe, was developed. The principle of the method consisted in removing the substrate, following enzymatic digestion, by precipitation with ammonium sulfate and determining the tosylarginine in solution. The enzymatic digestion was carried out in 20 per cent methyl Cellosolve because of the slight solubility of TABe in water. The procedure was as follows: 0.2 ml. of a 0.1 M solution of TABe in methyl Cellosolve was added to a digestion mixture containing 0.5 ml. of tris(hydroxymethyl)aminomethane (Tris) buffer (pH 9) and 0.3 ml. of enzyme solution in a 12 ml. glass centrifuge tube. At the end of digestion time, 4 ml. of cold saturated ammonium sulfate solution were added, the mixture was allowed to stand for 30 minutes, and the precipitated TABe removed by centrifugation. Blanks were prepared by reversing the addition of ammonium sulfate and TABe. 1 ml. aliquots of the supernatant fluids were diluted to 25 ml. with water and the optical density was determined at 230 mμ in a Beckman spectrophotometer with photomultiplier attachment. The difference between the blank and test sample represents the tosylarginine liberated by enzymatic digestion. Tosylarginine has an ultraviolet absorption spectrum with a 230 mμ maximum and a millimolar extinction of 12.05. The sensitivity of the method may be seen from a standard curve obtained with 0.1 to 0.8 γ of crystalline trypsin (Fig. 1).

The assay of LEe esterase was modified from the original method (9) by separating the lysine formed during digestion from the substrate. To 1 ml. of the digestion mixture, identical with the one described previously, 10 ml. of acetone were added and the mixture was allowed to stand for 30 minutes. The precipitated lysine, after separation by centrifugation and decantation of the supernatant fluid, was dissolved in 2 ml. of water. 2 ml. of formaldehyde (37 per cent neutralized to phenolphthalein pink)

[1] Obtained through the courtesy of the American National Red Cross from Dr. T. D. Gerlough of E. R. Squibb and Sons.

[2] Obtained through the courtesy of the Lederle Laboratories Division, American Cyanamid Company.

[3] The fibrinolytic assay (10) appears largely to measure the plasminogen activator (11), while the casein assay (12) measures the proteolytic enzyme plasmin.

were added, and the mixture was titrated with 0.05 N sodium hydroxide with a 1 ml. micro burette.

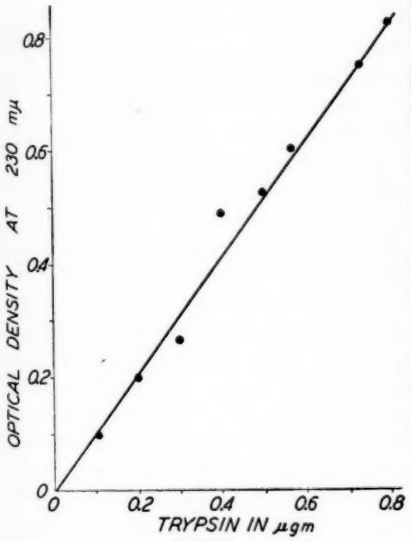


FIG. 1

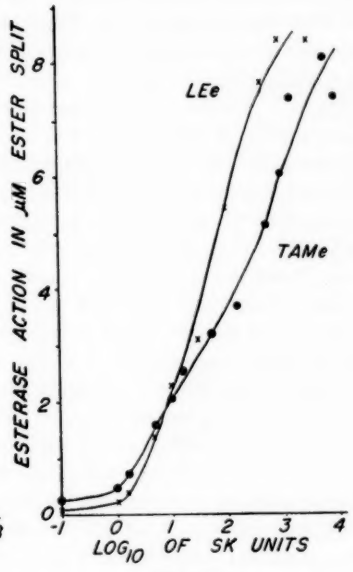


FIG. 2

FIG. 1. Standard curve for trypsin assay by the photometric TABe method. 30 minutes digestion at 37°. The method is as described in the text.

FIG. 2. LEe and TAMe esterase activity of plasminogen preparation (15 γ of nitrogen per ml.) activated by several concentrations of SK (expressed as $\log_{10}$ of SK units per ml.). LEe esterase and TAMe esterase activity was measured as described in the text and expressed as micromoles of ester split, per 30 minutes at 37° per ml.

Results

Evidence for Two Factors in Plasminogen

Previous studies had demonstrated that plasmin preparations attacked esters of the basic amino acids, arginine and lysine, but, upon purification of plasminogen and subsequent activation, the ratio of attack of arginine to lysine esters varied from preparation to preparation (9). Preliminary heat denaturation studies had suggested the presence of two enzyme precursors; the precursor of an enzyme capable of attacking LEe alone, along with the precursor of an enzyme capable of attacking both TAMe and LEe. Additional evidence for two factors in plasminogen preparations is as follows.

Activation Studies—Activation of plasminogen preparations by varying concentrations of SK revealed that the LEe and TAMe esterase activities bear different relations to the SK concentration (Fig. 2).

Heat Denaturation of Plasminogen Preparations—Heat denaturation studies of plasminogen preparations at selected pH values revealed different stabilities for the precursors of the LEe esterase and TAMe esterase (Fig. 3). Shown in Fig. 3 are heat denaturation studies of a plasminogen preparation at pH 2.0 and 9.0. The precursor of the TAMe esterase was more stable at pH 9.0, whereas the situation was reversed at pH 2.0. Heating plasminogen to 100° at pH 2 for prolonged periods resulted, upon activation, in an ever increasing ratio of LEe esterase to TAMe esterase activity. Occasionally, after 90 per cent or more of the initial activity was destroyed, preparations were obtained which, upon activation, contained only LEe esterase activity. These observations suggest the presence of the precursor of an enzyme with its activity limited to lysine substrates. Support for this view was obtained when fresh SK was added to heated plasmin preparations (Table III) which resulted in an increase in LEe esterase action with no increase in the TAMe esterase. Heating plasminogen to 50° at pH 9, for periods long enough to destroy 60 per cent or more of the initial activity, resulted in preparations which, upon activation, exhibited a constant ratio of TAMe esterase to LEe esterase activity of approximately 4. These latter observations suggested the presence of the precursor of a second enzyme capable of splitting TAMe at 4 times the rate of LEe.

Heat Denaturation of Plasmin Preparations—Heat denaturation studies of SK-activated plasminogen preparations revealed different stabilities for the TAMe and LEe esterases (Fig. 4). Shown in Fig. 4 are the stabilities for the TAMe and LEe esterases at 37° in the presence of 100 and 1000 units of SK. Only the stability of the LEe esterase was related to SK concentration, the LEe esterase being more stable in the presence of 1000 units of SK than in the presence of 100 units. The TAMe esterase stability appeared to be unaffected by SK concentration.

Inhibition Studies Relating LEe Esterase to Plasminogen Activator, and TAMe Esterase to Proteolytic Enzyme Plasmin

Previous studies had demonstrated that LEe and TAMe were striking competitive inhibitors of plasmin (9). In the following studies, the inhibition of the SK activation of plasminogen preparations by these synthetic substrates was investigated. The method used was to measure and compare the inhibition of enzyme activity when the synthetic substrate was added before and after SK activation. Inhibition of activation was apparent when the addition of inhibitor before SK activation resulted in a greater inhibition.

Inhibition of Activation of TAMe Esterase-Proteolytic Enzyme—LEe was observed to be a very striking inhibitor of activation of the TAMe esterase. Activation of the proteolytic enzyme was similarly inhibited, suggesting that the TAMe esterase and the enzyme responsible for casein proteolysis

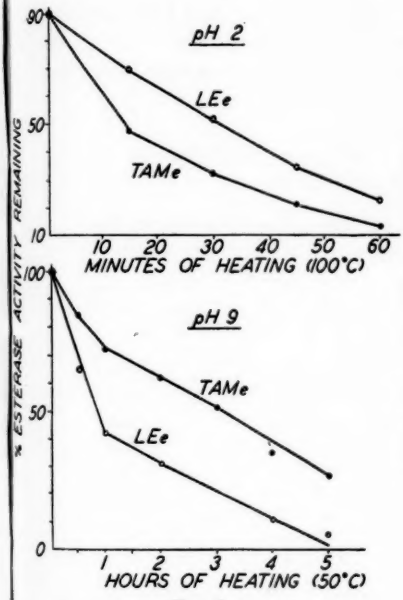


FIG. 3

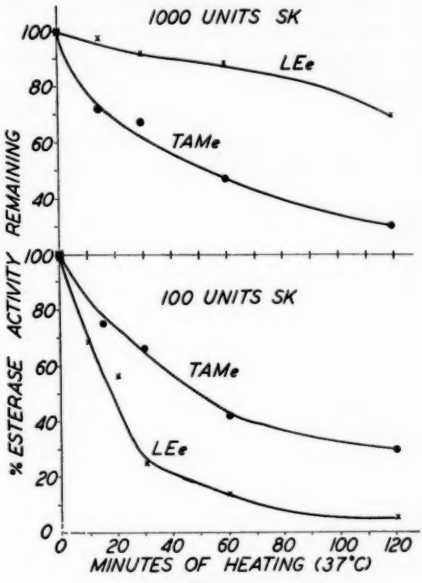


FIG. 4

FIG. 3. Heat stability of plasminogen preparation. Plasminogen preparation (20 γ of nitrogen per ml.) was heated at 100° at pH 2 (0.01 M hydrochloric acid) and to 50° at pH 9 (Tris buffer, 0.01 M). Samples were withdrawn at several times, activated with 1000 units of SK, and assayed for TAMe and LEe esterase activity. The results are expressed as per cent of original activity remaining.

FIG. 4. Heat stability of SK-activated plasminogen preparation. Plasminogen preparation (20 γ of nitrogen per ml.) activated with 1000 and 100 units of SK per ml. Incubated at 37°, and assayed for TAMe and LEe esterase activity at several times. The results are expressed as per cent of original esterase activity remaining.

were identical. TAMe, on the other hand, was a poor inhibitor to the activation of the enzyme splitting TAMe and casein. Data in Table I compare the inhibition of the TABe esterase activity by LEe, lysine, and ornithine and arginine when these inhibitors were added before or 5 minutes after SK activation. LEe was by far the most effective inhibitor of activation. Arginine had no inhibiting effect.

Since TAMe and LEe are effective competitive inhibitors in the proteo-

lytic assay (9), studies of the inhibiting effect of the synthetic substrates on the activation of the proteolytic enzyme required the removal of the esters prior to the final assay. This was accomplished by precipitating the activated enzyme with 1 M sodium chloride at pH 2, a technique which precipitated the enzyme quantitatively and left the esters in the supernatant fluid. Assays carried out in this fashion revealed that LEe inhibited the activation of the enzyme responsible for both proteolytic and TAMe esterase activity (Table I). TAMe at a concentration of 0.02 M did not

TABLE I

Comparison of Various Inhibitors on SK Activation of Plasmin

The values for the assays are given in per cent inhibition.

Inhibitor		Inhibitor present during enzyme assay			
		TABe assay			
		Added before SK		Added 5 min. after SK	
	M				
LEe	0.005	75.5		7.0	
"	0.04	96.6		43.4	
Lysine	0.04	73.0		0.0	
Ornithine	0.04	75.2		0.0	
Arginine	0.1	0.0		0.0	
		Inhibitor removed before enzyme assay			
		TAMe assay		Proteolytic assay (casein)	
		Added before SK	After SK	Before SK	After SK
LEe	0.02	67	5	60	5
TAMe	0.02	0	0	0	0

inhibit the activation of the TAMe esterase or proteolytic enzyme. At higher concentrations of TAMe, however, inhibition of activation could be demonstrated and accounts in part for the "substrate inhibition" previously noted (9).

Inhibition of Activation of LEe Esterase—Neither TAMe nor LEe inhibited the activation of the LEe esterase. LEe added before or 5 minutes after SK activation yielded identical substrate titration curves. TAMe added before SK had the same inhibiting effect as when added 5 minutes after SK, indicating that TAMe acted only as a competitive inhibitor to the LEe esterase but had no effect on activation.

When the plasminogen activator was estimated by the fibrinolytic

assay (11), the addition of LEe, lysine, and ornithine before or after the addition of SK resulted in an identical inhibition, suggesting that these substances did not interfere with the formation of the activator, yet were inhibitors to its action. The inhibition of the plasminogen activator by lysine and ornithine has been reported by Müllertz (13).

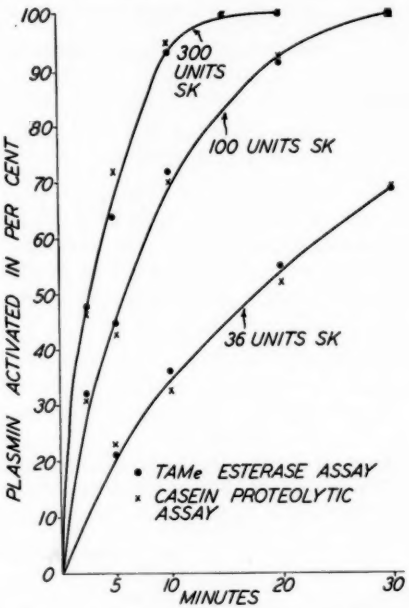


FIG. 5. Kinetics of plasmin activation. Plasminogen preparation (14 γ of nitrogen per ml.) was activated by 36, 100, and 300 units of SK per ml. at 25°. Activation stopped at several time intervals by the precipitation technique described in the text. TAMe esterase and casein proteolytic activity are expressed in per cent of total activity available.

Similarities in behavior in these inhibition studies relate the LEe esterase to the plasminogen activator, and identify the TAMe esterase with the proteolytic enzyme. The observation that LEe is the most effective inhibitor for the activation of the TAMe esterase-proteolytic enzyme (plasmin) is additional evidence for this view. Further confirmation was obtained through observations on a spontaneously active human plasmin preparation.[4] Following the addition of SK to this spontaneously active preparation, a marked increase in LEe esterase activity and of plasminogen

[4] Human plasma Fraction III_2, obtained through the courtesy of Dr. D. M. Surgenor of Harvard University.

activator (fibrinolytic assay) was observed with little change in the TAMe esterase or proteolytic activity.

TABLE II

Effect of Addition, Inactivation (Precipitation Technique), and Readdition of SK on Activation of Plasmin (TAMe Esterase-Proteolytic Enzyme), and of Plasminogen Activator (LEe Esterase)

Procedure	Plasmin assays		Activator assays		SK assay (Christensen (10)) units
	TAMe, μmole ester hydrolyzed	Casein units*	LEe, μmole ester hydrolyzed	Fibrinolytic units†	
I. Plasminogen	1.0	0.5	0.0	0	0
II. (I) + SK	10.3	3.9	10.0	3200	49,600
III. Ppt. of (II) with 1 M NaCl, pH 2	9.4	3.3	3.1	80	120
IV. (III) + SK	7.6	2.6	8.9	3200	24,000
V. Ppt. of (IV) with 1 M NaCl, pH 2	4.5	1.6	4.2	60	120
VI. (V) + SK	3.9	1.3	7.9	1600	24,800

* Remmert and Cohen (12).
† Christensen (10).

TABLE III

Effect of Addition, Inactivation (Heating Technique), and Readdition of SK on Activation of Plasmin (TAMe Esterase-Proteolytic Enzyme), and of Plasminogen Activator (LEe Esterase)

Procedure	Plasmin assays		Activator assays		SK assay (Christensen (10)) units
	TAMe, μmole ester hydrolyzed	Casein units	LEe, μmole ester hydrolyzed	Fibrinolytic units	
I. Plasminogen	2.5	0.2	0.5	0	0
II. (I) + SK	12.4	3.6	19.4	3200	4000
III. (II) heated to 100° for 15 min., pH 2	7.1	3.0	1.6	60	5
IV. (III) + SK	6.3	2.3	9.0	1600	1200

Mechanism of Activation of Plasmin (TAMe Esterase) and of Plasminogen Activator (LEe Esterase)

The true kinetics of plasminogen conversion to plasmin, following the addition of SK, were not observed unless the activation was stopped by removal of SK prior to estimation of plasmin activity. This was accomplished by a 2-fold reprecipitation of the activated plasmin at pH 2 with 1 M sodium chloride, a procedure which inactivated SK and stopped further

activation. By using this procedure at several time intervals, first order kinetics were observed for the activation of plasmin as measured either by the TAMe esterase or the casein assay (Fig. 5), and are in agreement with the observation of Christensen (3).

The kinetics of activation of plasminogen activator could not be studied in these experiments, since its activity, when measured either by the LEe esterase or the fibrinolytic assay, required the presence of SK. The dependence of LEe esterase and fibrinolytic assay activity on the presence of SK was demonstrated by experiments involving the removal of previously added SK, followed by the fresh addition of SK. From the data shown in Tables II and III, removal of SK, either by heating or precipitation, caused a prompt fall in plasminogen activator, while the addition of fresh SK produced an immediate rise of this enzyme. No such effect was noted on the TAMe esterase-proteolytic enzyme (plasmin). The activation of plasminogen activator appears to be instantaneous. The amount of activator formed and the stability of the activator (Fig. 4) were observed to be a function of SK concentration. Our observations with the fibrinolytic assay are in agreement with those of Wasserman (5).

It is concluded from these activation experiments that plasmin (TAMe esterase) is activated in enzymatic fashion, whereas the plasminogen activator (LEe esterase) is reversibly activated by SK in stoichiometric fashion.

DISCUSSION

The following schemes, similar to that proposed by Müllertz and Lassen (7), summarize the conclusions of our observations:

$$\text{Plasminogen proactivator} + \text{SK} \rightleftarrows \underset{\text{(LEe esterase)}}{\text{plasminogen activator}}$$

$$\text{Plasminogen} \xrightarrow[\text{activator}]{\text{plasminogen}} \underset{\text{(TAMe esterase-proteolytic enzyme)}}{\text{plasmin}}$$

Our work, by demonstrating the reversibility of this reaction, has provided new evidence for the stoichiometric interaction of SK with a plasma factor to form a plasminogen activator. Two possibilities remain to explain this interaction. SK may remove an inhibitor, or it may itself combine with the proactivator to form the activator.

In the enzymatic conversion of plasminogen to plasmin, we have confirmed the first order kinetics of the conversion. Identification of the enzymatic agent in this conversion as an LEe esterase is suggested by demonstration of the inhibition of plasminogen activation by LEe.

The enzymatic activation of plasminogen by plasminogen activator bears some similarity to the activation of trypsinogen by trypsin. The

activation of trypsinogen by trypsin has been shown to involve the splitting of a lysine peptide bond (14). The identification of plasminogen activator as an enzyme capable of splitting only lysine esters suggests that the activation of plasminogen also involves the splitting of a lysine peptide bond. It is of interest that trypsin can activate both human and bovine plasminogen (15).

The variability in resistance of different animal plasminogens to SK activation is well known (16). Bovine plasminogen is completely resistant to SK activation (16). Recent studies relate this lack of activation to the inability of SK to form a plasminogen activator in bovine plasma or bovine plasminogen preparations. In the presence of traces of human plasma or human plasminogen preparations, SK readily "activates" bovine as well as other animal plasminogens (7, 11). Since this latter phenomenon occurs in the fibrinolytic assay, in which the bovine fibrinogen substrate is heavily contaminated with plasminogen, a measure of the human plasminogen activator rather than the plasmin is obtained. It should be emphasized, however, that the clot is dissolved by bovine plasmin, which is the fibrinolytic enzyme, and not by the activator being measured.

The observation of the presence in human plasma of the precursor of an enzyme capable of rapidly activating plasminogen suggests that this substance may bear a relation to the natural mechanism of plasminogen activation.

SUMMARY

The activation of human plasminogen by streptokinase involves two steps: the "stoichiometric" interaction of streptokinase with a plasma factor to form an activator and the enzymatic activation of plasminogen by this activator. The plasminogen activator has the properties of an enzyme splitting lysine esters alone, and plasmin is a proteolytic enzyme capable of splitting arginine and lysine esters.

We gratefully acknowledge the assistance of Mr. John Wachman and Miss Mary Schmidt in these studies.

BIBLIOGRAPHY

1. Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, **28,** 559 (1945).
2. Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, **12,** 1 (1947).
3. Christensen, L. R., *J. Gen. Physiol.*, **28,** 380 (1945).
4. Ratnoff, O., *J. Exp. Med.*, **87,** 211 (1948).
5. Wasserman, A. E., *Arch. Biochem. and Biophys.*, **41,** 158 (1952).
6. Geiger, B. W., *J. Immunol.*, **69,** 597 (1952).
7. Müllertz, S., and Lassen, M., *Proc. Soc. Exp. Biol. and Med.*, **82,** 264 (1953).

8. Kline, D. L., *J. Biol. Chem.*, **204,** 949 (1953).
9. Troll, W., Sherry, S., and Wachman, J., *J. Biol. Chem.*, **208,** 85 (1954).
10. Christensen, L. R., *J. Clin. Invest.*, **28,** 163 (1949).
11. Sherry, S., *J. Clin. Invest.*, **33,** 1054 (1954).
12. Remmert, L. F., and Cohen, P. P., *J. Biol. Chem.*, **181,** 431 (1949).
13. Müllertz, S., *Proc. Soc. Exp. Biol. and Med.*, **85,** 326 (1954).
14. Davie, E. W., and Neurath, H., *Biochim. et biophys. acta*, **11,** 442 (1953).
15. Kocholaty, W., Ellis, W. W., and Jensen, H., *Blood*, **7,** 882 (1952).
16. Cliffton, E. E., and Cannamela, A. D., *Proc. Soc. Exp. Biol. and Med.*, **77,** 305 (1951).

STUDIES ON THIAMINE ANALOGUES

IV. NEOPYRITHIAMINE AND OXYNEOPYRITHIAMINE. A COMPARISON OF THEIR EFFECTS IN VIVO AND IN VITRO*

By LEOPOLD R. CERECEDO and STEPHEN EICH

(*From the Department of Biochemistry, Fordham University, New York, New York*)

(Received for publication, September 15, 1954)

Investigations previously reported from this laboratory have indicated a difference in the ability of two thiamine antagonists, oxythiamine and neopyrithiamine,[1] to inhibit the phosphorylation of thiamine *in vitro*. ATP-thiamine phosphokinase preparations from rat liver and rat intestine have been shown to be very easily inhibited by NPT, but not by OB_1 (1, 2). A consideration of these results suggested the possibility that the presence of an amino group in position 4 of the analogue pyrimidine moiety might be necessary for inhibition of the above systems. With this in view, we have prepared the 4-hydroxy derivative of NPT, oxyneopyrithiamine, and have compared its effects with those of NPT on the rat liver thiamine phosphorylase system, as well as *in vivo* in the mouse.

EXPERIMENTAL

Materials

Cocarboxylase, neopyrithiamine, 2-methyl-4-hydroxy-5-methoxymethylpyrimidine, and 2-methyl-3-β-hydroxyethylpyridine were generously supplied by Dr. Karl Folkers of Merck and Company, Inc., Rahway, New Jersey. Sodium adenosinetriphosphate was a commercial preparation obtained from the Schwarz Laboratories, Inc., Mt. Vernon, New York.

The Swiss mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. α-Carboxylase was prepared from wheat germ according to the method of Singer and Pensky (3). The rat liver ATP-thiamine phosphokinase employed was the "precipitated HCl" fraction of Leuthardt and Nielsen (4).

The 2-methyl-4-hydroxy-5-bromomethylpyrimidine hydrobromide was prepared from 2-methyl-4-hydroxy-5-methoxymethylpyrimidine by treatment with 10 per cent hydrobromic acid in glacial acetic acid according to the method employed by Cline *et al.* (5) for the preparation of 2-methyl-4-

* Aided, in part, by contract No. AT(30-1)-1056 between the Atomic Energy Commission and Fordham University.

[1] The following abbreviations are used throughout: B_1, thiamine; OB_1, oxythiamine; NPT, neopyrithiamine; ONPT, oxyneopyrithiamine; ATP, adenosinetriphosphate.

amino-5-bromomethylpyrimidine. The product decomposed without melting at 228°.

Oxyneopyrithiamine (2-Methyl-3-β-hydroxyethyl-N-[(2-methyl-4-hydroxypyrimidyl-5-methyl)]pyridinium Bromide)—To a solution of 600 mg. of 2-methyl-3-β-hydroxyethylpyridine in 3 ml. of isopropyl alcohol 186 mg. of 2-methyl-4-hydroxy-5-bromomethylpyrimidine were added, and the mixture was allowed to stand at room temperature. The crystals, which began to form after a few hours, were filtered on the following day. They were washed, first with isopropyl alcohol and then several times with chloroform. It was necessary to wash the product thoroughly to remove traces of the starting pyridine compound which could be detected when the product was chromatographed on paper. The compound melted at 193–195° with decomposition. It gave a positive diazosulfanilic acid reaction and a negative thiochrome test.

$C_{14}H_{18}N_3O_2Br$.	Calculated.	C 49.5, N 12.3, H 5.32
	Found.	" 49.1, " 12.1, " 5.33

Methods

Thiamine Phosphorylation—Phosphorylations were studied in the manner previously described (1), and α-carboxylase was again employed for the estimation of thiamine phosphorylation.

Preparation of Mice—Male Swiss mice were placed on a thiamine deficient diet consisting of purified casein (Labco) 25, sucrose 53, hydrogenated vegetable oil (Crisco) 10, lard 5, salt mixture (U. S. P. XIV) 5, and roughage (Ruffex) 2 per cent. It contained, per kilo of diet, riboflavin 10 mg., pyridoxine 10 mg., calcium pantothenate 100 mg., α-tocopherol 40 mg., vitamin A 67,000 units, vitamin D (Drisdol) 5000 units, and choline chloride 1.5 gm. The animals began to lose weight after approximately 11 days on the diet. When they were eating 1 gm. of food or less per day, they were injected subcutaneously with 1 or 2 γ of thiamine per day for 1 week. At this point, the mice were considered standardized, and the injections of the thiamine analogues were begun.

Results

Phosphorylation of Thiamine—Upon comparison of neopyrithiamine and oxyneopyrithiamine as inhibitors of the ATP-thiamine phosphokinase, a difference was observed. The phosphorylation of thiamine by the rat liver preparation was not affected by the presence of oxyneopyrithiamine (Table I). Neopyrithiamine, on the other hand, when added at the same molar levels as ONPT, caused an almost complete inhibition of the system.

Mouse Experiments—Two separate experiments were performed. The level of thiamine injected daily was 1 γ in the first experiment. A striking

difference between the effects of NPT and ONPT was evident (Table II). The mice receiving NPT developed the typical symptoms of polyneuritis and died approximately 2 days later. In contrast, those injected with ONPT at the same molar ratio (analogue to thiamine) exhibited no such

TABLE I

Effects of Neopyrithiamine and Oxyneopyrithiamine on Rat Liver ATP-Thiamine Phosphokinase

The complete system contained 0.5 ml. of enzyme preparation, 0.1 ml. of 1 M $MgCl_2$, 3 mg. of ATP, 2.0 ml. of 0.1 M phosphate, pH 7.4, 10 γ of B_1. Total volume, 3.4 ml. Incubated 3 hours at 38°. 1 ml. removed, after boiling, for estimation of thiamine diphosphate.

Additions	Thiamine diphosphate
	γ
None	2.5
250 γ ONPT	2.7
500 " "	2.6
313 " NPT	0.3
625 " "	0.2

TABLE II

Effects of Neopyrithiamine and Oxyneopyrithiamine in Vivo

Group No.	Mice per group	Compounds injected per day	Molar ratio (analogue to B_1)	Mean survival time after date of administration of analogues	Incidence of polyneuritis
				days	*per cent*
1	3	1 γ B_1		40	0
2	4	1 " " + 6.5 γ NPT	5	7.5	100
3	4	1 " " + 5.0 " ONPT	5	43	0
4	5	1 " " + 25.0 γ ONPT	25	28	0
5	5	1 " " + 50.0 " "	50	24	0

effects and, moreover, maintained weights comparable to those of the control group.

Several mice in Groups 4 and 5 (see Table II) lost weight, developed a rather severe diarrhea, and died, whereas others in these groups displayed a tolerance to the compound. Thus, administration of higher levels of ONPT gave rise to a more complicated situation. On the basis of these results, it is difficult to attribute definitely such an effect to an antagonism brought about by the thiamine analogue. We are inclined to consider a toxic effect of the pyridine moiety as the responsible agent, especially in view of the poor physical condition of the mice, resulting from the small

XUM

amount of vitamin administered. The lack of well being of the animals was manifest from the unkempt appearance of the control group and their relatively short survival period.

In view of these inconclusive results, the experiment was repeated, but the level of thiamine injected was raised to 2 γ per day, the molar ratios of analogue to thiamine remaining the same as in the first experiment. The results of this experiment appear to be more conclusive (Fig. 1). Although the mice all received 2 γ of thiamine, the effect of neopyrithiamine was the same as in the previous experiment. The group which received ONPT at a 5:1 molar ratio (analogue to vitamin) (Curve C) showed a growth rate

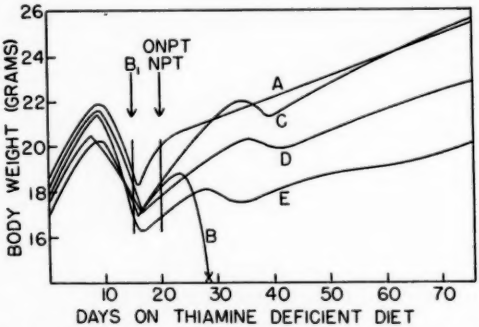


FIG. 1. Growth curves of mice showing effects of oxyneopyrithiamine and neopyrithiamine. Curve A, mean body weight of four animals receiving 2 γ of B_1 per day; Curve B, four animals receiving 2 γ of B_1 plus 13.5 γ of NPT, at a molar ratio, analogue to B_1, of 5:1; Curve C, eight animals receiving 2 γ of B_1 plus 10 γ of ONPT, 5:1 ratio; Curve D, seven animals receiving 2 γ of B_1 plus 50 γ of ONPT, 25:1 ratio; Curve E, five animals receiving 2 γ of B_1 plus 100 γ of ONPT, 50:1 ratio.

resembling that of the control group, while those injected with ONPT at molar ratios of 25:1 and 50:1 (Curves D and E) lagged behind the control group, although they continued to gain weight.

Thus, the effect of increasing the amount of thiamine from 1 to 2 γ per day, while the molar ratio of analogue to vitamin was kept constant, resulted in no change with regard to NPT. In the case of ONPT, however, the mice were able to resist the deleterious effect observed in the first experiment. These results are an indication that the initial effect of ONPT was probably due to the pyridine moiety rather than to an antithiamine action. This view seems to be borne out by consideration of the growth curves of the animals which received ONPT. In each case, shortly after administration of the analogue, there was a distinct drop in the growth rate, followed by a slow increase. These depressions may have resulted from an initial toxicity of the compound to which the animals were able to adapt

themselves. In spite of this, however, it is obvious that growth is still dependent upon the level of ONPT administered.

DISCUSSION

It is clear from the data presented that the inhibitory nature of neopyrithiamine is dependent to a large degree upon the presence of its pyrimidyl 4-amino group. Its substitution by a hydroxyl group yielded a substance which did not inhibit the phosphorylation of thiamine *in vitro* and did not produce polyneuritis.

It is of interest here to compare the effects of oxythiamine and oxyneopyrithiamine. Both of these compounds contain a 4-hydroxy group. They differ in that the thiazole ring of OB_1 has been replaced by a pyridine ring in ONPT. Neither will inhibit cocarboxylase synthesis by the liver phosphokinase, nor do they cause the typical polyneuritic symptoms when administered to mice. However, oxythiamine is an effective thiamine antagonist *in vivo* (6, 7). Oxythiamine, at a 25:1 molar ratio (analogue to thiamine), will cause an antithiamine effect in mice, while experiments reported here indicate no such effect of ONPT at this level, or even at a 50:1 ratio. Thus, the substitution of the thiazole for the pyridine ring in oxythiamine results in the disappearance of this toxicity. It would seem that oxythiamine is sufficiently related to thiamine to function as an antagonist, while ONPT, further removed structurally from the vitamin, is now outside the range of antagonistic effectiveness.

SUMMARY

1. Oxyneopyrithiamine, the 4-hydroxypyrimidyl derivative of neopyrithiamine, has been prepared.

2. Oxyneopyrithiamine, in contrast to neopyrithiamine, is ineffective as an inhibitor of rat liver ATP-thiamine phosphokinase.

3. Comparative studies in mice indicate that oxyneopyrithiamine does not function as an inhibitor of thiamine.

4. These findings demonstrate the necessity of the 4-aminopyrimidyl group in neopyrithiamine for the inhibitory properties of the analogue.

BIBLIOGRAPHY

1. Eich, S., and Cerecedo, L. R., *J. Biol. Chem.*, **207**, 295 (1954).
2. Cerecedo, L. R., Eich, S., and Bresnick, E., *Biochim. et biophys. acta*, **15**, 144 (1954).
3. Singer, T. P., and Pensky, J., *J. Biol. Chem.*, **196**, 375 (1952).
4. Leuthardt, F., and Nielsen, H., *Helv. chim. acta*, **35**, 1196 (1952).
5. Cline, J. K., Williams, R. R., and Finkelstein, J., *J. Am. Chem. Soc.*, **59**, 1052 (1937).
6. Eusebi, A. J., and Cerecedo, L. R., *Science*, **110**, 162 (1949).
7. Cerecedo, L. R., Soodak, M., and Eusebi, A. J., *J. Biol. Chem.*, **189**, 293 (1951).

AMINO ACID STUDIES IN THE TRANSFORMATION OF PROTEINS OF THE HEN'S EGG TO TISSUE PROTEINS DURING INCUBATION*

By C. O. RUPE† and CHESTER J. FARMER†

(From the Department of Biochemistry, Northwestern University Medical School, Chicago, Illinois)

(Received for publication, July 19, 1954)

Abderhalden and Kempe (1) analyzed the hen's egg for glutamic acid, glycine, and tyrosine on the 1st, 10th, and 21st day of incubation. Their results indicated that the amounts of these acids remained constant throughout the incubation period. Investigations carried out by Calvery (2–4), Plimmer and Lowndes (5), Schenck (6), and Sendju (7, 8) indicate that variations occur in the amounts of arginine, histidine, lysine, tryptophan, and tyrosine contained in the egg as the chick embryo develops. However, there exists wide disagreement as to the nature of these changes.

Studies on the distribution of amino acids in the incubated hen's egg have not been reported since 1932, therefore the more modern techniques of amino acid analysis have not been applied. Furthermore, the distribution of several of the amino acids in the egg has never been investigated at all. Because of the disagreement among previous investigators and of the limited number of amino acids studied, a study by means of microbiological assay has been undertaken to review the distribution of arginine, histidine, lysine, tryptophan, and tyrosine in the white, yolk, and embryo of the developing hen's egg. The amino acids aspartic acid, glutamic acid, glycine, isoleucine, leucine, methionine, phenylalanine, threonine and valine, not previously studied in the various components (white, yolk, embryo), have been included in the present investigation.

Materials

White Leghorn eggs obtained from a certified hatching strain were used throughout the study. They were collected during the months of February, March, April, and May, 1948, from hens kept on a diet of constant composition. The eggs were shipped to the laboratory within 3 days after laying, weighed to the nearest 0.1 gm., and placed in a warm air incubator

* Taken in part from a thesis submitted by C. O. Rupe in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Northwestern University, 1950.

† Veterans Administration Research Hospital, 333 East Huron Street, Chicago 11, Illinois.

at 38° with a relative humidity of 60 to 70 per cent which, according to Murray (9) and Barott (10), is the optimum.

Two eggs were removed for assay purposes at the beginning of the incubation period and approximately every 48 hours thereafter until the end of 400 hours. The white, yolk, and embryo of each egg were separated and like components pooled. The shell and shell membrane were not included in the study. The separation was accomplished in the early stages of development (0 to 120 hours) by first removing the shell and shell membrane from over the embryo and making an incision in the yolk sac just outside of the marginal vein. The embryo and the extra-embryonic tissue were lifted from the yolk, washed in distilled water, blotted dry with filter paper, and weighed. The remainder of the egg was frozen in a mixture of solid carbon dioxide and methyl Cellosolve. The shell was then removed and the yolk and white were separated and weighed while frozen.

Eggs incubated more than 120 hours were first frozen, the shell was removed, and the three components were separated. This procedure permitted the removal of the allantoic sac and its fluid with the embryo without mixing with the yolk or white. Each component was weighed, thoroughly ground in a mortar, lyophilized, and stored in the refrigerator. Before being assayed, the lyophilized materials were pulverized and dried over anhydrous calcium chloride. Samples ranging from 0.2 to 1.5 gm. were hydrolyzed with 10 per cent HCl as described by Stokes *et al.* (11). For the estimation of tryptophan and tyrosine, alkaline hydrolysates (11) were used. Since amino acids undergo complete racemization during alkaline hydrolysis, a factor of 2 was used in calculating the amounts of tryptophan and tyrosine. No attempt was made to correct for any racemization or destruction of amino acids that might occur during acid hydrolysis.

The hydrolysates were adjusted to pH 6.8 to 7.0, diluted to 100 ml., filtered, and stored in the refrigerator under toluene as stock solutions. The assays were run on 1:4 dilutions of these solutions.

Microbiological assays (12, 13) were used for the determination of all amino acids except glycine. *Streptococcus faecalis*[1] was used for the estimation of arginine, histidine, isoleucine, leucine, lysine, methionine, threonine, tyrosine, and valine. Aspartic acid and glutamic acid were determined with *Lactobacillus arabinosus*, phenylalanine with *Lactobacillus casei*, and tryptophan with *Lactobacillus delbrueckii*. Glycine was determined by the ninhydrin method of Alexander *et al.* (14).

[1] Cultures of *L. arabinosus* 17-5, *L. casei* 9595, *L. delbrueckii* LD5, and *S. faecalis* 9790 were obtained originally from the American Type Culture Collection, Georgetown University.

RESULTS AND DISCUSSION

The values for the various amino acids are expressed as the amount present in the separate components. These data are plotted against time and the lines of regression for each component are drawn by inspection. The total amount of the different amino acids in the whole egg is the sum of the respective amino acids in the white, yolk, and embryo at each time interval. No values are given for the embryo under 72 hours of incubation because of the small quantity of solid embryonic material available.

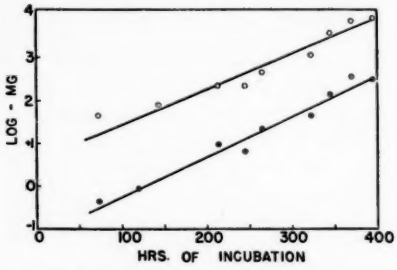
TABLE I

Rate of Accumulation of Amino Acids by Chick Embryo

Amino acid	Values of b^* × 10^4
Arginine	88
Aspartic acid	91
Glutamic "	67
Glycine	92
Histidine	85
Isoleucine	83
Leucine	100
Lysine	81
Methionine	73
Phenylalanine	87
Threonine	82
Tyrosine	106
Tryptophan	93
Valine	84
Embryo growth	75

* b = log of the quantity of amino acids, in mg., accumulated per hour.

The fact that the weight of the chick embryo increases exponentially (15) suggests that the increase of an amino acid in the embryo would also follow an exponential curve. By plotting the logarithms of the dry weight or the values of the various amino acids against time, one obtains a straight line (Fig. 1) showing that the accumulation of an amino acid and the increase in weight follow the same pattern. The rates at which the weight increases and the amino acids accumulate are given by the slopes of these lines. The values vary from 0.0067 to 0.0106 (Table I). According to covariance analysis, this variation is no greater than would be expected from random sampling, a result which indicates that the amino acid accumulation by the embryo not only follows the same pattern as the weight increase, but, between the 72nd and 400th hour of incubation, also occurs at the same rate.



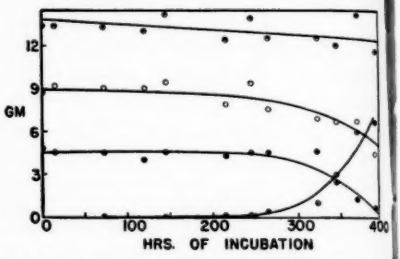
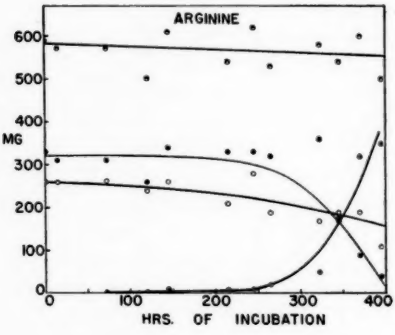
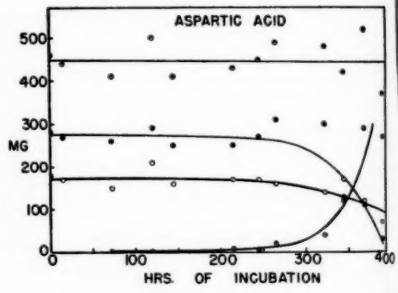
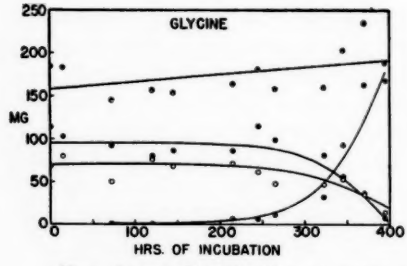


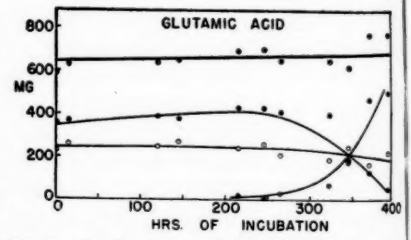
FIG. 1. Log of the weight increase (○) and aspartic acid increase (⊙) of the chick embryo during incubation.

FIG. 2. Dry weight distribution in the hen's egg during incubation. Whole egg (◓), white (●), yolk (○), embryo (⊙).









FIGS. 3 TO 6. Distribution of amino acids in the hen's egg during incubation. Whole egg (◓), white (●), yolk (○), embryo (⊙).

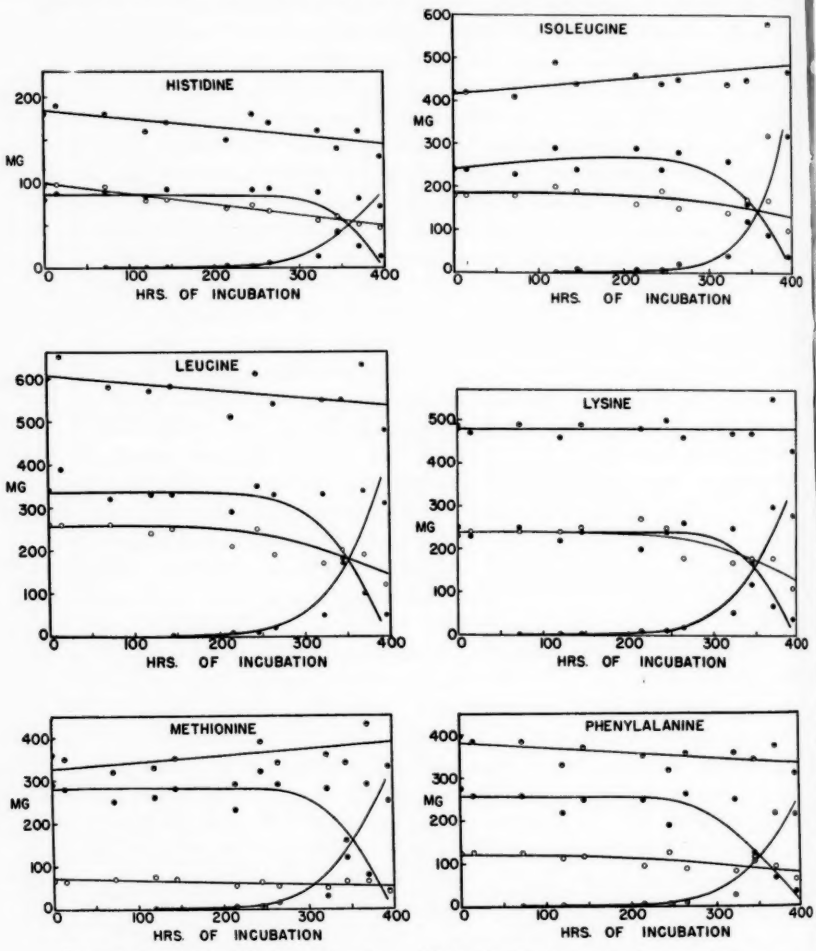
It is common knowledge that the egg loses solids during incubation. The graph in Fig. 2, representing the distribution of dry weight in the developing hen's egg, shows this loss to be about 1.5 gm. Murray (9) and Tangl and von Mituch (16) have studied the nature of the solids lost and have estimated that at least 90 per cent of this loss is due to lipide oxidation. Fiske and Boyden (17) state in this connection that not more than 6 per cent of the organic material oxidized was protein and that 96 per cent of the egg protein is converted to tissue protein. This, along with Needham's (18) statement that only 11 mg. of nitrogen are excreted by the embryo during development, leads one to suspect that the larger portion of the catabolized material is non-protein in nature and that only a small fraction of the total weight loss is due to destruction of amino acids.

The question as to which amino acids are destroyed and which are synthesized can be answered by studying the amounts of the various amino acids in the whole egg as incubation progresses. The total content of each amino acid was computed for the whole egg and plotted against time (Figs. 3 to 16). The best possible straight line as determined by the method of least squares was drawn through the points. The critical ratio of the slope to the standard error of the slope, commonly known as the "t" value, was calculated for each line, in order to determine to what extent the slope differed from zero. Those cases in which the t value was such that there were less than two chances in 100 that the slope was zero were considered to be significant. According to this criterion, valine and histidine showed a loss, while the other amino acids showed no significant change.

During the first 400 hours of incubation, 43 per cent of the yolk substance is utilized by the embryo. The yolk furnishes most of the materials needed for the first 250 hours of development, as evidenced by the early decline in weight and in amino acids. The amino acid content, even though lower than that of the white, is adequate both in type and in quantity for the early phase of embryonic growth. It can be seen from the individual graphs (Figs. 3 to 16) that the utilization of the yolk amino acids does not follow a uniform pattern, but that these substances appear to be utilized at independent rates. By the end of 400 hours of incubation, 77 per cent of the glycine, 59 per cent of the aspartic acid, 50 per cent of the histidine, 31 per cent of the methionine, and 11 per cent of the tryptophan have disappeared. The ratios of the various amino acids to each other as they occur in the unincubated yolk and as they disappear vary markedly, thus indicating preferential utilization of individual yolk amino acids by the embryo.

There is no decline in the weight of the white or of its amino acids until the 250th hour, at which time the embryonic growth has become so rapid

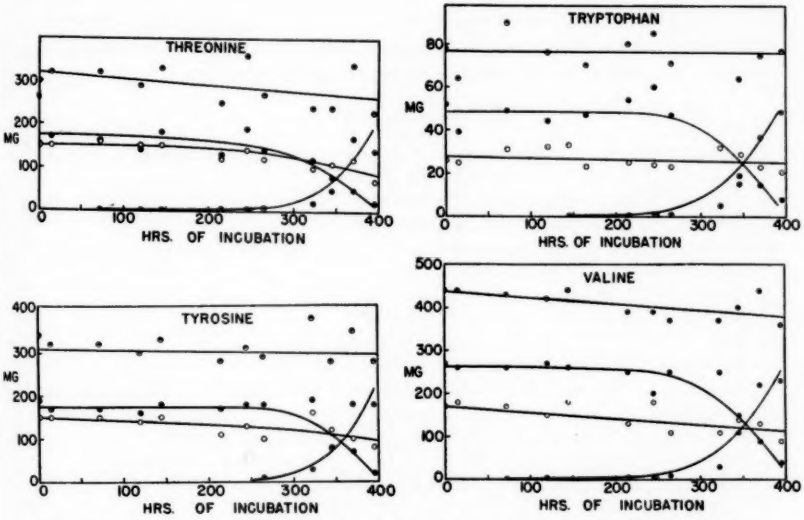
that the yolk can no longer adequately supply the necessary amino acids. From the 250th to 400th hour, the white loses 86 per cent of its material.



FIGS. 7 TO 12. Distribution of amino acids in the hen's egg during incubation

Over the same period of time, the individual amino acids decrease by approximately the same proportion. The amino acid transfer between the white and embryo appears to be a simple migration, the amino acids decreasing in the same ratio as they exist in the white at the beginning of

incubation. This is in agreement with a reported (19, 20) observation of a constant ratio between the albumin and ovomucoid in the white throughout incubation. In this connection, it is interesting to note that the amino acids exist in the embryonic tissue in the same relationship as they do in the white, in that those which occur in the largest amounts in the white also occur in the largest amounts in the embryo. This strongly suggests that the amino acids incorporated in the egg white proteins are present in the proper type and quantity for the development of embryonic tissue.



FIGS. 13 TO 16. Distribution of amino acids in the hen's egg during incubation

This would not necessarily have to be the case if the embryo were either synthesizing or catabolizing amino acids.

The process of the chick development appears to be one of transfer and incorporation of existing amino acids of the egg proteins into embryonic tissue. From the evidence presented, it appears that the embryo builds itself from the amino acids present in the egg at the beginning of the incubation period. The possibility of synthesis is not excluded, but, if it occurs, the extent is limited, since there is no increase in the quantity of any one amino acid over and above that existing at the beginning of incubation. The possibility of catabolism of the amino acids appears more likely, since there is evidence that valine and histidine do decrease during incubation.

SUMMARY

A study of the distribution of fourteen amino acids has been made on the white, yolk, and embryo during incubation of the hen's egg. Between the 72nd and 400th hour of incubation, all amino acids accumulate in the embryo at the same rate. The results indicate that, for the first 250 hours of incubation, the yolk is the sole source of amino acids for the embryo and that preferential utilization of these amino acids occurs. From the 250th until the 400th hour, the white supplies the major portion of amino acids needed by the embryo. These amino acids are utilized in the same ratio as they exist in the white. With the exception of histidine and valine, which show a slight decrease, the amounts of the several amino acids in the egg remain constant throughout incubation.

BIBLIOGRAPHY

1. Abderhalden, E., and Kempe, M., *Z. physiol. Chem.*, **53**, 398 (1907).
2. Calvery, H. O., *J. Biol. Chem.*, **83**, 231 (1929).
3. Calvery, H. O., *J. Biol. Chem.*, **83**, 649 (1929).
4. Calvery, H. O., *J. Biol. Chem.*, **87**, 691 (1930).
5. Plimmer, R. H. A., and Lowndes, J., *Biochem. J.*, **21**, 254 (1927).
6. Schenck, E. G., *Z. physiol. Chem.*, **211**, 111 (1932).
7. Sendju, Y., *J. Biochem.*, Japan, **5**, 391 (1925).
8. Sendju, Y., *J. Biochem.*, Japan, **7**, 175 (1927).
9. Murray, H. A., Jr., *J. Gen. Physiol.*, **9**, 1 (1925–26).
10. Barott, H. G., *U. S. Dept. Agr., Tech. Bull. 553* (1937).
11. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
12. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
13. Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159**, 273 (1945).
14. Alexander, B., Landwehr, G., and Seligman, A. M., *J. Biol. Chem.*, **160**, 51 (1945).
15. Murray, H. A., Jr., *J. Gen. Physiol.*, **9**, 39 (1925–26).
16. Tangl, F., and von Mituch, A., *Arch. ges. Physiol.*, **121**, 437 (1908).
17. Fiske, C. H., and Boyden, E. A., *J. Biol. Chem.*, **70**, 535 (1926).
18. Needham, J., Chemical embryology, Cambridge (1931).
19. Bywaters, H. W., *J. Physiol.*, **45**, p. xl (1912–13).
20. Bywaters, H. W., and Roue, W. B., *J. Physiol.*, **46**, p. xxxiii (1913).

GLUCOSAMINE AND THE ACTION OF INSULIN*

By ARNE N. WICK, DOUGLAS R. DRURY, HENRY I. NAKADA, HARRY N. BARNET, AND TOSHIKO N. MORITA

(From the Scripps Metabolic Clinic, La Jolla, and the Department of Physiology, University of Southern California School of Medicine, Los Angeles, California)

(Received for publication, October 11, 1954)

Insulin increases the transfer rate of glucose (1), galactose (2, 3), and mannose (4) from the extracellular fluid into the cells of the extrahepatic tissues. Its action, however, is highly specific. Compounds closely related to glucose such as fructose (5), sorbitol (6), gluconic acid (7), glucuronic acid (7), and 3-methylglucose (7) cannot enter the muscle cells of the living rabbit to any measurable extent and the administration of insulin does not alter this. With regard to galactose (3), we found evidence of competition between it and glucose in entering into the reaction that transfers the sugars intracellularly, a reaction which is accelerated by insulin. A high concentration of glucose depresses the transfer rate of galactose. We were unable to test whether this effect is reciprocal, that is, whether a high concentration of galactose will depress the action of insulin on glucose, since it is difficult to determine glucose accurately in the presence of a high concentration of galactose.

In the present work we report evidence that another glucose analogue, D-glucosamine, is acted upon by insulin. This compound should be useful in investigating the problem of competitor effect on the action of insulin on glucose, since it can be determined chemically in the presence of glucose and lends itself easily to separation from glucose by ion exchange resins.

Methods

The glucosamine hydrochloride used in these studies was purchased from the Eastman Kodak Company and purified before use by treatment with charcoal and recrystallization three to five times (8). Although the final white crystalline product had the correct physical constants, a neutral reducing substance was shown to be present when the material was passed over a cation exchange column (Duolite C-3). When calculated as glucose, the neutral fraction appeared to account for 1 to 3 per cent of the weight of the injected material.

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service. The insulin used in this work was contributed by the Lilly Research Laboratories.

Determination of Free Plasma Glucosamine—The plasma was deproteinized by dilution with absolute alcohol (1:20). Free glucosamine in the supernatant fluid was determined by the Schloss (9) modification of the Elson and Morgan colorimetric method with two slight variations as suggested by Blix (10). The acetylacetone concentration of Reagent A was increased to 5 ml. per 50 ml. in order to reduce color from glucose and free amino acids and the *p*-dimethylaminobenzaldehyde was increased to 1.6 gm. per 30 ml. of concentrated HCl and 30 ml. of absolute alcohol to give a more constant glucosamine color at higher concentrations of glucosamine. Recoveries of known amounts of glucosamine from plasma were close to 100 per cent up to concentrations of 500 mg. per cent.

Determination of Plasma Glucose in Presence of Glucosamine—The neutral reducing sugar in the alcohol supernatant fluid was separated from glucosamine by adsorbing the basic substances on an 8 × 50 mm. column containing Duolite C-3 ion exchange resin regenerated to the acid state. The reducing sugars were determined in the effluent by the method of Miller and Van Slyke (11) and were generally found in the first three or four 3 ml. fractions. Recovery of glucose in the effluent was about 100 per cent. Glucosamine could be eluted from the column with 0.5 N NaCl or HCl as the elutriant, but recoveries were not as good as those obtained by the direct colorimetric method.

Animal Procedure—Non-fasted female rabbits were eviscerated and nephrectomized as previously described (12). These animals were maintained by the injection of glucose. In those animals injected with tagged glucose the uniformly labeled form of glucose was used. A tracheal cannula was inserted during the operation, and this served for subsequent collection of expired CO_2. The method for determining the radioactivity in the CO_2 was the same as that used before (1). The glucosamine was injected intravenously as the hydrochloride. After allowing 30 minutes for body mixing, the first plasma sample was taken for glucose and glucosamine analysis. In those rabbits given insulin, the hormone was administered in maximal amounts (20 units) after the taking of the first plasma sample and each hour thereafter. Plasma samples were usually taken at hourly intervals during the experiment.

Results

The glucosamine concentration in the plasma samples taken 30 minutes after its injection may be used for calculating its immediate volume of distribution. The values for Experiments 1 to 10 given in Fig. 1 are 21, 25, 14, 20, 17, 23, 23, 23, 25, and 20 per cent of the body weight, respectively. These values suggest that at this time the glucosamine is largely in the extracellular compartment. For each animal the plasma concentration at various times thereafter has been calculated relative to the

30 minute concentration by assigning the value of 100 to it. These values are presented in Fig. 1. It is apparent that glucosamine, like glucose, enters the muscle cells to a limited amount in the absence of insulin. The administration of insulin, however, definitely accelerates the transfer rate of glucosamine.

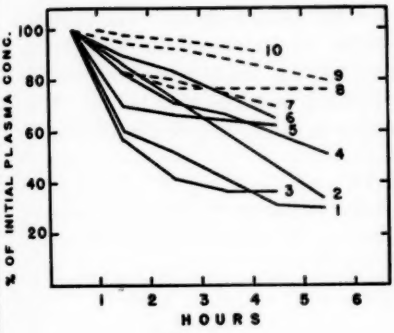


FIG. 1

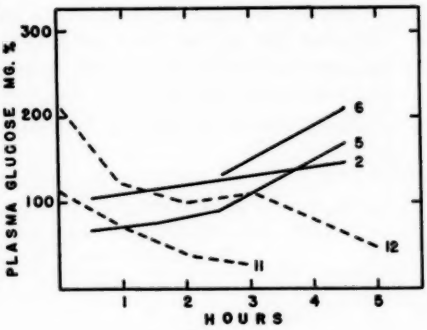


FIG. 2

FIG. 1. The effect of insulin on the disappearance rate of injected D-glucosamine hydrochloride from plasma. All rabbits injected intravenously at zero hour with 0.5 gm. per kilo except that in Experiment 9 which received 1 gm. per kilo. 30 minutes allowed for mixing before taking initial plasma sample. Solid line, insulin-treated animals; broken line, no insulin.

FIG. 2. The effect of a single dose of D-glucosamine hydrochloride injected at zero hour (0.5 gm. per kilo) on the plasma glucose concentration. In Experiments 2, 5, 6, and 11 the glucose was injected intravenously by constant infusion at the rate of 200 mg. per kilo per hour. In Experiment 12 the rate of infusion was 356 mg. per kilo per hour. Solid line, glucosamine hydrochloride administered; broken line, no glucosamine.

In Fig. 2 are data showing the effect of a single glucosamine injection on the disappearance rate of glucose from plasma in insulinized animals. These results are of particular interest because it has been our experience that a glucose infusion rate of 300 mg. per kilo per hour in the maximally insulinized animal is about the minimal infusion rate that is compatible with survival although at a hypoglycemic level. Ordinarily it requires 500 mg. of glucose per kilo per hour to maintain the blood sugar level at 100 mg. per cent (13). Two experiments have been included to show the rapid drop in blood sugar concentration to the convulsive levels when amounts less than 500 mg. per kilo per hour are infused without the simultaneous injection of glucosamine at the beginning of the experiment. In contrast to these experiments, the administration of a single dose of glucosamine decreases the transfer rate of glucose; thus even at the low glucose infusion rate of 200 mg. per kilo, despite insulin in maximal amounts,

there is a constant rise in plasma sugar. These results show that D-glucosamine inhibits the intracellular transfer of glucose.

It might be suggested that this effect of glucosamine might result if glucosamine were converted to glucose in the blood stream. A simple calculation will show that this cannot explain the result. In order to have such a rise in blood sugar in maximally insulinized animals, 500 mg. of glucose per kilo per hour would have to be administered. The rabbits received 200 mg. per kilo per hour. If the conversion of glucosamine to glucose were to account for the difference, 300 mg. per kilo per hour would need to be so changed, or a total of 1200 mg. per kilo in 4 hours. The animals were given 420 mg. of glucosamine per kilo, practically one-third

TABLE I

Per Cent of Expired CO_2 from Injected Glucose in Insulin-Treated Eviscerated Nephrectomized Animals Given D-Glucosamine Hydrochloride As Single Injection at Start of Experiment (1 Gm. Per Kilo)

Period	Glucosamine-treated			Control*	
	Rabbit 380	Rabbit 385	Rabbit 390	Rabbit 1	Rabbit 2
hr.					
1st	4			11	6
2nd	5	10		27	16
3rd	8	10		37	22
4th	10	10		39	30
5th	14	11	19	43	39
6th	15	12	23	39	39
7th	17		29	43	42
8th	20		29	43	45

* Data taken from a previous publication (17).

the amount needed if conversion were the way by which these results were obtained. In Table I are data presenting the effect of glucosamine injection on the oxidation of glucose. We have reported previously that in the eviscerated animal maximally treated with insulin about 50 per cent of the expired CO_2 comes from the injected glucose after the 5th or 6th hour of continuous glucose infusion. It is seen that in the glucosamine-treated animals these values are reduced in all cases below 29 per cent. If glucosamine is preventing the transfer of plasma glucose, results of this nature would be expected as a secondary effect. These data then parallel the transfer rate inferred from the data given in Fig. 2.

DISCUSSION

D-Glucosamine definitely responds to the action of insulin. In so doing, it inhibits the intracellular transfer of glucose. Evidence for this is the

low rate of plasma glucose disappearance following a single injection of D-glucosamine. This may be due to a competition effect between the two compounds or to a blocking action of glucosamine. The latter effect could occur if the amine is not metabolized, or only slowly so. The use of tagged glucosamine will be necessary to determine its rate of oxidation. It is of interest in this connection that Harpur and Quastel (14) found that D-glucosamine is phosphorylated at the expense of adenosinetriphosphate in the presence of aqueous extracts of an acetone powder of beef brain. It was further shown that glucose and glucosamine compete for the phosphorylating enzyme involved. Brown (15) later showed that crystalline yeast hexokinase also phosphorylated glucosamine. Since insulin increases the intracellular transfer of these compounds, it might suggest that this reaction is a primary one in the action of insulin; however, galactose, which also responds to insulin, apparently is not phosphorylated by hexokinase (16) obtained from muscle.

SUMMARY

Insulin increases the intracellular transfer of D-glucosamine in the extrahepatic tissues. Glucosamine in this respect resembles glucose, mannose, and galactose. The intracellular transfer of glucosamine is associated with a substantial reduction in the transfer rate of glucose. The possible causes for the inhibition of the glucose transfer are discussed.

BIBLIOGRAPHY

1. Wick, A. N., Drury, D. R., Bancroft, R. W., and MacKay, E. M., *J. Biol. Chem.*, **188,** 241 (1951).
2. Levine, R., Goldstein, M. S., Huddlestun, B., and Klein, S. P., *Am. J. Physiol.*, **163,** 70 (1950).
3. Wick, A. N., and Drury, D. R., *Am. J. Physiol.*, **173,** 229 (1953).
4. Drury, D. R., and Wick, A. N., *Am. J. Physiol.*, **177,** 535 (1954).
5. Wick, A. N., Sherrill, J. W., and Drury, D. R., *Diabetes*, **2,** 465 (1953).
6. Wick, A. N., and Drury, D. R., *Am. J. Physiol.*, **166,** 421 (1951).
7. Drury, D. R., and Wick, A. N., *Diabetes*, in press.
8. Purchase, E. R., and Braun, C. E., *Org. Syntheses*, **26,** 36 (1946).
9. Schloss, B., *Anal. Chem.*, **23,** 1321 (1951).
10. Blix, G., *Acta chem. Scand.*, **2,** 467 (1948).
11. Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, **114,** 583 (1936).
12. Drury, D. R., *Am. J. Physiol.*, **111,** 289 (1935).
13. Wick, A. N., and Drury, D. R., *Am. J. Physiol.*, **167,** 359 (1951).
14. Harpur, R. P., and Quastel, J. H., *Nature*, **164,** 693 (1949).
15. Brown, D. H., *Biochim. et biophys. acta*, **7,** 487 (1951).
16. Colowick, S. P., in Sumner, J. B., and Myrbäck, K., The enzymes, New York, **2,** pt. 1 (1951).
17. Wick, A. N., and Drury, D. R., *Am. J. Physiol.*, **174,** 445 (1953).

THE AMINO ACID REQUIREMENTS OF MAN

IX. THE PHENYLALANINE REQUIREMENT*

By WILLIAM C. ROSE, BYRON E. LEACH,† MINOR J. COON,† AND G. FREDERICK LAMBERT‡

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois)

(Received for publication, October 29, 1954)

As explained elsewhere (1), only eight amino acids are indispensable components of the food of man. Recently, a quantitative investigation of the L-tryptophan requirement was described, and 0.25 gm. was adopted *tentatively* as the minimal daily intake which is capable of maintaining nitrogen equilibrium (2). The reasons for this conclusion were discussed in detail. The present paper is a continuation of the program designed to establish the minimal needs of adult man for each of the essential amino acids and is concerned with the L-phenylalanine requirement.

EXPERIMENTAL

The general procedures followed in the conduct of the experiments were described fully in earlier papers of this series (3, 2). Healthy young men served as the subjects. The nitrogen of the diets was furnished in the form of mixtures of purified amino acids, to which glycine and urea (*cf.* (4)) were added as extra sources of nitrogen for the synthesis of the non-essentials. Most of the energy of the rations was derived from wafers composed of corn-starch, sucrose, unsalted butter fat (which had been melted and centrifuged to remove particles of protein), corn oil, a salt mixture, and a baking powder prepared in this laboratory. Additional calories, as needed in individual cases, were obtained by the consumption of appropriate amounts of sucrose and butter fat. Vitamins were supplied in the form of a fish liver oil concentrate and pills composed of crystalline compounds supplemented with a butyl alcohol extract of Wilson's liver powder 1:20.

* Aided by grants from the Nutrition Foundation, Inc., and the Graduate College Research Fund of the University of Illinois.

† The experimental data in this paper are taken from theses submitted by Byron E. Leach and Minor J. Coon in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the University of Illinois. Present address of Byron E. Leach, School of Medicine, Tulane University, New Orleans, Louisiana. Present address of Minor J. Coon, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

‡ Present address, Nutrition Research Department, Abbott Laboratories, North Chicago, Illinois.

XUM

The reader is referred to Paper I of this series (3) for the composition of the salt mixture, the baking powder, and the vitamin pills.

Six experiments were conducted in determining the L-phenylalanine requirement. Two of these, which illustrate the range of values obtained, are detailed below. Both experiments, like the first quantitative tryptophan tests (2), were carried out at an early stage in our program when some uncertainty still existed regarding the possible rôle of histidine. In

TABLE I

Composition and Daily Intakes of Amino Acid Mixtures

Component	Mixture 8		Mixture 30		Mixture 135	
	As used	N content	As used	N content	As used	N content
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Valine	8.56*	1.02	8.56*	1.02	8.56*	1.02
Leucine	4.91	0.52	4.91	0.52	9.82*	1.05
Isoleucine	6.16*	0.66	6.16*	0.66	6.16*	0.66
Methionine	3.68*	0.35	3.68*	0.35	3.68*	0.35
Threonine	6.16*	0.72	6.16*	0.72	6.16*	0.72
Phenylalanine	4.29	0.36	1.50	0.13	2.20*	0.19
Tryptophan	1.85	0.25	1.85	0.25	0.25	0.03
Lysine monohydrochloride	7.69	1.18	7.69	1.18	1.50	0.23
Histidine monohydrochloride monohydrate			3.29	0.66		
Glycine	14.52	2.71	12.22	2.28	18.87	3.52
Urea	4.29	2.00	4.29	2.00	4.29	2.00
	62.11	9.77†	60.31	9.77†	61.49	9.77‡

* Racemic acids.

† Of the nitrogen contributed by this mixture, 1.20 gm. were derived from the D isomers of valine, isoleucine, and threonine.

‡ Of the nitrogen contributed by this mixture, 1.72 gm. were derived from the D isomers of valine, leucine, isoleucine, and threonine.

consequence of this fact, histidine was included in the food during part of each test. Later events proved this precaution to be unnecessary, since dietary histidine is not essential for nitrogen equilibrium in man (5, 6). Four other measurements of the phenylalanine requirement, the details of which are omitted for the sake of brevity, involved the use of histidine-free rations throughout. The results thereby obtained will be revealed later in this paper.

The first subject, H. I. E., received initially a daily diet composed of amino acid Mixture 8 (Table I), Wafers III (the composition of which has been described in an earlier paper (2)), 183.8 gm. of additional sucrose, and

69 gm. of additional butter fat. Cellu flour, to provide a residue in the alimentary tract, and the usual vitamins (*cf.* (3)) were administered apart from the basal ration. As shown in Table I, amino acid Mixture 8 contained only the eight essentials plus glycine and urea. The daily allotment furnished 9.77 gm. of nitrogen, of which 1.20 gm. were derived from the D isomers of valine, isoleucine, and threonine. As is our practice, the amino acid mixture was consumed in aqueous solution flavored with filtered lemon juice. The entire diet supplied 9.99 gm. of nitrogen, of which 0.22 gm. was of an unknown nature. This was derived largely from the starch and in

TABLE II

Phenylalanine Requirement of Man, Subject H. I. E.

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
5	70.3	9.99	8.28	1.01	+0.70	4.29 gm. L-phenylalanine
7	71.7	9.99	8.71	0.92	+0.36	2.86 " "
4	72.1	9.99	10.40	0.87	−1.28	No L-phenylalanine
5	71.7	9.99	8.84	0.93	+0.22	2.30 gm. L-phenylalanine
4	72.1	9.99	8.58	1.09	+0.32	2.00 " "
9	72.6	9.99	8.88	0.84	+0.27	1.00 " "
9	73.5	9.99	8.97	0.90	+0.12	0.80 " "
5	72.6	9.99	9.43	0.93	−0.37	0.70 " "

* During the first four periods, the amino acid mixture contained only the eight essentials plus glycine and urea; thereafter, it also furnished 3.29 gm. of histidine monohydrochloride monohydrate daily. This was introduced at the expense of an equivalent amount of nitrogen in the form of glycine.

lesser amounts from the butter fat, lemon juice, and liver concentrate present in the vitamin pills. The daily energy intake from all sources amounted to 3850 calories. One-third of the amino acid solution and other dietary components was consumed at each meal.

The results of the experiment upon Subject H. I. E. are summarized in Table II. Throughout, as alterations were made in the phenylalanine intake, the nitrogen content of the food was maintained at a constant level by appropriate adjustments in the amount of glycine present in the amino acid mixture. With a daily consumption of 4.29 gm. of L-phenylalanine, positive balance was established promptly. For the 5 day period, an average daily retention of 0.70 gm. occurred. Furthermore, positive balance continued when the daily phenylalanine intake was decreased to 2.86

gm. During the third period, exclusion of phenylalanine from the food induced a strong negative balance, as was anticipated, and this condition was reversed immediately by the return to the ration of 2.30 gm. daily of the missing amino acid.

At this point, as indicated above, data obtained in another experiment appeared to suggest that histidine might exert a beneficial effect upon nitrogen balance. Later events proved this interpretation to be incorrect. However, at the moment, the inclusion of histidine seemed advisable despite the positive nitrogen balances obtained without this amino acid. Therefore, during the last four periods, Subject H. I. E. received each day 3.29 gm. of histidine monohydrochloride monohydrate in place of an equivalent amount of nitrogen in the form of glycine. Our entire experience with amino acid diets warrants the affirmation that the alteration in question did not affect the outcome of the test. The data in Table II show that positive nitrogen balance was maintained successively by daily intakes of 2.00, 1.00, and 0.80 gm. of L-phenylalanine. On the contrary, further reduction of the intake to 0.70 gm. resulted in a distinctly negative balance. Thus, the findings show clearly that the minimal daily L-phenylalanine requirement of Subject H. I. E. was 0.80 gm.

Subject M. J. C. served in two phenylalanine tests which were separated by an interval of almost 1 year. The first of these involved the use throughout of a diet which furnished 3.29 gm. of histidine monohydrochloride monohydrate daily. By the time the second test upon this subject was undertaken, histidine had been classified definitely as a non-essential amino acid; consequently, it was excluded from the ration.

The initial diet of Subject M. J. C. was composed of amino acid Mixture 30 (Table I), Wafers IV (*cf.* (7)), 142 gm. of additional sucrose, and 16.1 gm. of additional butter fat. As customary, Cellu flour and the vitamins were administered apart from the basal ration. The daily allotment of Mixture 30 furnished 9.77 gm. of nitrogen, of which 1.20 gm. were derived from the D isomers of valine, isoleucine, and threonine. The entire diet supplied 10.08 gm. of nitrogen, of which 0.31 gm. was of an unknown nature. The energy intake from all sources amounted to 3575 calories.

The results of the first test upon Subject M. J. C. are summarized in the upper half of Table III. As will be observed, he came into positive balance readily upon a daily intake of 1.50 gm. of L-phenylalanine. When, however, the daily dose was reduced to 0.80 gm., the amount found to be the minimal requirement of Subject H. I. E., a pronounced negative balance ensued. During the three succeeding periods, M. J. C. showed nitrogen retention with intakes of 1.20 and 1.10 gm. of the amino acid and almost exact equilibrium (−0.02 gm.) with an intake of 1.00 gm. On the basis of these observations, one might be tempted to conclude that

1.00 gm. represented his minimal daily requirement. The close approximation of the nitrogen intake and output at this phenylalanine level was responsible for the extension of the period to 14 days. It was hoped that thereby a definite decision might be warranted regarding the adequacy of the dosage. However, during the period, the subject was slightly in positive balance for 6 days and slightly in negative balance for 8 days. In

TABLE III

Phenylalanine Requirement of Man, Subject M. J. C.

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
5	66.2	10.08	8.71	1.09	+0.28	1.50 gm. L-phenylalanine
6	66.2	10.08	9.83	1.09	−0.84	0.80 " "
6	66.2	10.08	8.89	0.93	+0.26	1.20 " "
14	65.8	10.08	9.11	0.99	−0.02	1.00 " " †
4	65.8	10.08	8.69	0.92	+0.47	1.10 " "
7	66.2	10.08	8.78	0.98	+0.32	1.10 " DL-phenylalanine
Intermission						
8	64.0	10.08	8.47	1.33	+0.28	2.20 gm. DL-phenylalanine
5	64.4	10.08	8.51	1.38	+0.19	1.10 " L-phenylalanine
5	64.9	10.08	9.41	1.44	−0.77	1.10 " D-phenylalanine
7	64.4	10.08	8.91	1.38	−0.21	1.50 " "
6	64.9	10.08	8.71	1.32	+0.05	1.10 " DL-phenylalanine
4	64.9	10.08	8.29	1.32	+0.47	1.20 " "

* During the first six periods, the amino acid mixture furnished 3.29 gm. of histidine monohydrochloride monohydrate daily in addition to the eight essentials, glycine, and urea. During the last six periods the mixture was devoid of histidine.

† The subject was in positive balance during 6 days and in negative balance during 8 days of this period.

instances of this sort, as pointed out in Paper VII (2), the practice has been followed uniformly of designating as the minimal requirement the smallest quantity of an amino acid which is capable of inducing a *distinctly positive balance*, as measured by the average of a period of several days. In accordance with this principle, 1.10 gm. daily must be regarded as the minimal L-phenylalanine requirement of this subject.

The last period of the first test upon M. J. C. demonstrated that 1.10 gm. of DL-phenylalanine were capable of replacing an equal weight of the L isomer. Thus, the organism possessed the ability of inverting at least

0.55 gm. of D-phenylalanine daily. This is in striking contrast to the findings with DL-tryptophan (*cf.* (2)), which in adult man proved to be an unsatisfactory substitute for L-tryptophan.

The second test upon M. J. C. was designed to obtain further insight into the metabolic availability of D- and DL-phenylalanine. The initial diet was composed of amino acid Mixture 135 (Table I), Wafers IV (*cf.* (7)), 143.5 gm. of additional sucrose, and 16.4 gm. of additional butter fat. Cellu flour and the vitamins were again administered apart from the basal ration. Thus, the diet was essentially unchanged from that used in the first test, except with respect to the amino acid mixture. The latter (Mixture 135) contained DL- instead of L-leucine, and smaller amounts of L-tryptophan and L-lysine monohydrochloride than were present in Mixture 30 (Table I). Furthermore, it was devoid of histidine. The daily allotment of Mixture 135 furnished 9.77 gm. of nitrogen, of which 1.72 gm. were derived from the D isomers of valine, leucine, isoleucine, and threonine. The total nitrogen of the diet amounted to 10.08 gm., of which 0.31 gm. was of an unknown nature, and the energy content was 3575 calories.

In order to conserve a dwindling supply of L-phenylalanine, Subject M. J. C. was first brought into positive balance with DL-phenylalanine. For this purpose, 2.20 gm. were administered daily. As will be seen from the data in Table III, the average daily nitrogen retention during this period amounted to 0.28 gm. The DL-phenylalanine was then replaced by 1.10 gm. of L-phenylalanine, which amount had previously been shown to represent the subject's minimal daily requirement. Positive nitrogen balance continued, as was anticipated, despite the absence of histidine from the food. Of particular interest, however, are the results obtained during the last four periods of the test. The figures reveal the fact that the substitution of 1.10 gm. of D-phenylalanine for a like amount of the L isomer induced a strongly negative nitrogen balance. Furthermore, the loss of body nitrogen continued, though at a diminished rate, even when the intake of D-phenylalanine was raised to 1.50 gm. daily. Evidently, some of the D isomer was being inverted; otherwise the intensity of the deficiency would have been much more pronounced (*cf.* (8), and Table II, third period, of the present paper). During the penultimate period, the replacement of D-phenylalanine by 1.10 gm. of DL-phenylalanine induced a slightly positive balance, which became more pronounced during the final period when the daily intake of the racemic amino acid was increased to 1.20 gm.

On the basis of the above observations, one must conclude that the ability of Subject M. J. C. to invert D-phenylalanine was limited in magnitude or rate, and probably did not significantly exceed 0.55 gm. daily.

Entirely comparable findings were obtained in other individuals. In Paper VII (2) of this series, attention was called to the fact that Subject R. E. F. (Table V) manifested a negative nitrogen balance during a single period in which the diet contained 2.20 gm. of D-phenylalanine instead of the 2.40 gm. of DL-phenylalanine administered daily during all other periods. Therefore, in this case also, the subject was unable to invert a sufficient quantity of the D isomer to meet his minimal phenylalanine requirements. In two other subjects the administration of D-phenylalanine in amounts equal to or greater than their minimal L-phenylalanine needs induced strongly negative nitrogen balances. As far as our experience goes, this behavior is a general one. On the contrary, two subjects, in addition to M. J. C., were tested for their ability to maintain nitrogen equilibrium upon rations containing DL-phenylalanine in amounts equal to or slightly in excess of their minimal L-phenylalanine requirements. Each succeeded in doing so, and thereby demonstrated a capacity to invert at least 0.45 gm. of the D isomer daily. These findings substantiate the conclusion reached in the case of Subject M. J. C.; namely, that an upper limit exists for the inversion of the compound. In this respect, D-phenylalanine differs from the optical isomers of the other essential amino acids, one of which undergoes inversion even more readily, and six of which appear not to be inverted at all by man, in so far as one is able to determine by the nitrogen balance technique. Evidence in support of these relationships will be presented in subsequent papers.[1]

As pointed out above, the L-phenylalanine requirements of four additional subjects were determined. The conditions employed were entirely comparable to those already described, except that histidine-free diets were used throughout. The findings in all six individuals are summarized in Table IV. At a later date, when the sparing action of tyrosine on the phenylalanine requirement of man is considered, the data obtained in two of the tests (Subjects H. E. S. and R. L. S.) will be presented in full.

A foot-note to Table IV calls attention to the fact that the figure given for Subject R. R. C. may not represent his exact minimal requirement. With a dosage of 0.80 gm. of L-phenylalanine, this subject experienced an average daily loss of 0.36 gm. of nitrogen, and with the consumption of 1.00 gm. of the amino acid he showed an average daily retention of 0.14 gm. of nitrogen. Unfortunately, it became necessary to discontinue the experiment before the effect of 0.90 gm. daily could be tested. In view of

[1] The above statements in reference to the inversion of amino acids are not intended to minimize the possible significance of other factors which may have contributed to the observed results. It is well known that D-amino acids are less rapidly absorbed from the intestine (9) and are less efficiently retained by the kidneys (10) than are L-amino acids.

the very moderate positive balance obtained at the 1.00 gm. level, it seems extremely unlikely that a lower intake would have permitted nitrogen equilibrium.

The data in Table IV support an affirmation made in discussing the minimal tryptophan requirements of man (2); namely, that no correlation seems to exist between the body weights of the subjects and their needs for a particular amino acid. Also, as was the case with tryptophan, no relationship could be detected between the phenylalanine requirement and the body surface or creatinine output of the individual. This is a peculiar and unexpected fact which has been observed with each of the essential amino acids. Perhaps it indicates that the *mass* of active protoplasm is

TABLE IV

Summary of L-*Phenylalanine Experiments*

Subject	Body weight	Minimal daily requirement
	kg.	*gm.*
H. E. S.	65.2	0.8
M. J. C.	66.2	1.1
H. I. E.	70.3	0.8
R. L. S.	71.0	1.0
W. A. S.	71.7	0.9
R. R. C.	78.5	1.0 or less*

* With this subject, 0.8 gm. of L-phenylalanine per day yielded a distinctly negative nitrogen balance, and 1.0 gm. daily induced a moderately positive balance. Unfortunately, an intake of 0.9 gm. was not tested.

not the sole factor affecting the requirement of the organism for an amino acid.

With respect to the method of designating the minimal requirement of man for an amino acid, reasons have been given elsewhere (2) for the opinion that the use of the mean of the observed values and its probable error is not satisfactory. In investigations in which the number of experiments must of necessity be limited, and in which variability from one subject to another is to be anticipated, the most appropriate practice, it seems to us, is *to designate the highest value obtained as the tentative minimal requirement.* Consequently, 1.10 gm. are hereby proposed as the *tentative* L-phenylalanine requirement of adult human males. To allow for the possibility that other individuals who have not been tested may have still higher requirements, twice this tentative minimum, or 2.20 gm. of L-phenylalanine per day, may be regarded as a *safe* intake (*cf.* (2)). Experience with a larger number of young men may show that this quantity may be lowered with-

out sacrificing safety. Indeed, in most of the experiments which were involved in measuring the requirements of man for other amino acids, 2.40 gm. of DL-phenylalanine were included in the rations. If one assumes that approximately 0.55 gm. of the D isomer can be inverted, as was the case in Subject M. J. C., then the above quantity of DL-phenylalanine would be equivalent to 1.75 gm. of the L isomer. Twenty-eight subjects have now been maintained in positive nitrogen balance on daily intakes of 2.40 gm. or less of DL-phenylalanine, and no subject has been found with a higher requirement.

SUMMARY

Six quantitative experiments have been conducted upon normal young men for the purpose of determining their minimal L-phenylalanine requirements. Positive nitrogen balance was the criterion of dietary adequacy. The results demonstrate that the subjects required daily intakes of 0.80, 0.80, 0.90, 1.00, 1.00, and 1.10 gm., respectively. No relationship was observed between the minimal phenylalanine needs of the subjects and their body weights, body surfaces, or creatinine output.

Inasmuch as an investigation of this nature precludes the possibility of using a large number of subjects, and in view of the variability which one encounters in different individuals, it seems appropriate, in the interest of prudence, *tentatively* to designate the highest observed value, or 1.10 gm., as the minimal daily L-phenylalanine requirement of man.

In recognition of the possibility that other individuals who have not been tested may require still larger intakes of L-phenylalanine, provision should be made for this contingency in formulating human diets designed to exclude amino acid deficiencies. Twice the highest observed minimal requirement, or 2.20 gm. daily, is certainly a *safe* intake. Experience with a larger number of subjects may eventually warrant the adoption of a lower figure for this purpose. Emphasis should be placed on the fact that safe intakes are not synonymous with optimal intakes. The latter cannot be measured directly and may be quite high.

Experiments upon four individuals reveal that significant amounts of D-phenylalanine can be utilized by the human organism. The inversion of this amino acid appears to be limited to approximately 0.50 gm., or slightly more, per day. For this reason, D-phenylalanine is incapable of replacing a sufficient quantity of L-phenylalanine to meet the minimal requirement of man. On the contrary, DL-phenylalanine, when included in the food at a level equal to or slightly in excess of an individual's minimal L-phenylalanine requirement, appears to be almost as effective as the L isomer.

BIBLIOGRAPHY

1. Rose, W. C., Haines, W. J., and Warner, D. T., *J. Biol. Chem.*, **206**, 421 (1954).
2. Rose, W. C., Lambert, G. F., and Coon, M. J., *J. Biol. Chem.*, **211**, 815 (1954).
3. Rose, W. C., Johnson, J. E., and Haines, W. J., *J. Biol. Chem.*, **182**, 541 (1950).
4. Rose, W. C., Smith, L. C., Womack, M., and Shane, M., *J. Biol. Chem.*, **181**, 307 (1949).
5. Rose, W. C., Haines, W. J., Warner, D. T., and Johnson, J. E., *J. Biol. Chem.*, **188**, 49 (1951).
6. Rose, W. C., Haines, W. J., and Warner, D. T., *J. Biol. Chem.*, **193**, 605 (1951).
7. Rose, W. C., Coon, M. J., and Lambert, G. F., *J. Biol. Chem.*, **210**, 331 (1954).
8. Rose, W. C., Warner, D. T., and Haines, W. J., *J. Biol. Chem.*, **193**, 613 (1951).
9. Gibson, Q. H., and Wiseman, G., *Biochem. J.*, **48**, 426 (1951).
10. Crampton, R. F., and Smyth, D. H., *J. Physiol.*, **122**, 1 (1953).

NUCLEOTIDE METABOLISM

IV. THE PHOSPHORYLATION OF 5′-URIDINE NUCLEOTIDES BY CELL FRACTIONS FROM RAT LIVER*

By EDWARD HERBERT,† VAN R. POTTER, and YASUYUKI TAKAGI‡

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison, Wisconsin)

(Received for publication, July 26, 1954)

In previous work from this laboratory the occurrence of the mono-, di-, and triphosphates of uridine, guanosine, and cytidine in the acid-soluble fraction of liver and other tissues was established (2). As part of a program concerned with the significance of these compounds in nucleic acid synthesis (3, 4), investigations were undertaken to determine their rôle in oxidative phosphorylation. The present paper is concerned with the phosphorylation of the 5′-uridine nucleotides.

The study of the naturally occurring acid-soluble uridine nucleotides has a brief history. Following the discovery of UDPG[1] (5–7) and several other derivatives of UDP (8–10), a number of studies have been concerned (11–25) with the metabolism of these compounds in tissue extracts, but to our knowledge there has been no report of studies on the phosphorylation of UMP or UDP in terms of cell fractions isolated from homogenates.

The experiments reported here indicate that the adenosine nucleotides are preferential phosphate acceptors between uridine nucleotides and oxidative phosphorylation donors. In addition they demonstrated, apparently for the first time (1), the existence of an enzyme capable of phosphorylating UMP by direct reaction with ATP and showed that this enzyme is found in the supernatant fraction from the mitochondria. The mitochondria are unable to phosphorylate UMP, but rapidly catalyze a reaction between UDP and ATP in the absence of the supernatant fraction.

* A preliminary report has appeared (1). This work was supported in part by a grant (No. C-646) from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and in part by an institutional grant (No. 71) from the American Cancer Society.

† Postdoctorate Research Fellow of the National Heart Institute, United States Public Health Service.

‡ On leave from the Department of Biochemistry, Osaka City Medical School, Japan.

[1] The following abbreviations are used: AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates; UMP, UDP, UTP, and UDPG, uridine mono-, di-, and triphosphates and uridine diphosphate glucose; IP, inorganic or orthophosphate; PCA, perchloric acid.

EXPERIMENTAL

Cell Fractionation—Cell fractions prepared from the livers of male rats weighing 150 to 200 gm. were used as the source of enzymes in these experiments. After the animal was decapitated, the liver was quickly excised and placed in ice-cold isotonic NaCl. The tissue was then blotted dry and homogenized in ice-cold isotonic sucrose (0.25 M) with an all-glass Potter-Elvehjem type homogenizer. In the time-course experiments (Figs. 3 to 6 and Table III) in which the "cytoplasmic fraction" was used, the homogenate was centrifuged at 600 × *g* for 10 minutes to remove the nuclei. When more extensive cell fractionation was carried out, it was performed in isotonic sucrose according to the method of Schneider and Hogeboom (26). The nuclear and mitochondrial fractions were washed once in isotonic sucrose before use, and the fluffy layer which sediments on top of the mitochondria in the isolation procedure was swirled off after the washing. Except when the fluffy layer was treated as a separate fraction (Experiment 1, Table I, and Fig. 2) it was added to the supernatant liquid from the mitochondria. All of the fractionation procedures were carried out at 4°. The unit referred to in Tables II and III and Figs. 1 and 3 to 6 as mg. equivalents of the cell fractions corresponds to the mg. wet weight of liver.

Reaction Systems—The standard incubation mixture was made up so that each 3.0 ml. portion contained the following: 15 μmoles of potassium pyruvate, 15 μmoles of potassium glutamate, 6 μmoles of potassium fumarate, 15 μmoles of potassium phosphate buffer at pH 7.2, 9 μmoles of $MgCl_2$, and sufficient solid sucrose to give a final concentration of 0.25 M including the sucrose in the tissue preparation. When oxygen consumption was measured, the incubations were carried out in conventional Warburg flasks. When time studies on nucleotide conversions were performed, the incubations were carried out in 125 ml. Erlenmeyer flasks to insure a large enough surface area for gas exchange, because the initial volume in these cases was 13 or 16 ml. In such experiments the tissue, usually in a volume of 3.5 ml. of isotonic sucrose, was equilibrated at 30° for 2 minutes and then added to the standard incubation medium (in a volume of 9.5 or 12.5 ml.) which had been equilibrated at 30° for 10 minutes. After 15 to 20 seconds were allowed for mixing, a sample of 3.0 ml. was removed and immediately deproteinized. The incubations were carried out at 30° between pH 7.1 and 7.4. All of the figures for oxygen consumption reported here represent averages of determinations on duplicate flasks.

The enzyme reactions were stopped by the addition of 1.5 ml. of 1.5 N PCA to each 3.0 ml. sample to give a final concentration of 0.5 N PCA. These mixtures were centrifuged, and aliquots of the supernatant liquids were removed for orthophosphate determination by the method of Fiske

and Subbarow (27). Two analyses for orthophosphate were performed on each supernatant liquid. The remainder of the supernatant liquid was neutralized with 2 N KOH and allowed to stand in the refrigerator overnight to permit precipitation of the potassium perchlorate (28).

Analytical—The separation and quantitative analysis of the adenosine and uridine nucleotides in the supernatant liquids were performed by chromatography on manually operated Dowex 1 (X10, 200 to 400 mesh) formate anion exchange columns (0.78 sq. cm. × 3 cm.). The columns were prepared according to the procedure described previously by Hurlbert, Schmitz, Brumm, and Potter (28). In this laboratory the complete separation of the uridine and adenosine nucleotides is achieved ordinarily by the use of a two column chromatographic technique (28) which involves elution of the nucleotides from one formate column with a gradually increasing concentration of formic acid at pH 2 and then rechromatography of the more complex peaks so obtained on a second formate column with a gradually increasing concentration of ammonium formate at pH 5. In the method used here the resolving power of the two formate systems has been combined in a single chromatogram. Fig. 1 illustrates that use of this combined system results in separation of all of the nucleotides employed in these experiments. The method was as follows: After the neutralized supernatant liquids were put on the columns, elution was begun with six 5 ml. portions of water to remove nucleosides. The elution was continued with the solutions indicated below the chromatogram in the order shown. Thus, formic acid was used until ADP was off the column (by means of 2.5 N formic acid); then ammonium formate was employed to complete the chromatogram. Each 5 ml. portion eluted from the column is represented by a step in the chromatogram in Fig. 1, and the optical density reading of each portion at 260 mμ is plotted as the ordinate. The discontinuities (spaces) between the peaks in the chromatogram serve to emphasize that different solutions were used to elute each peak. The nucleotide composition of each peak was determined initially by comparing the position and shape of the peak with that of purified known compounds on this combined system, as indicated in Fig. 1, and by other means which are discussed in another section of this paper. The position of these peaks does not vary from one column to another or from one time to another provided the length of the column and the sample volume are the same in each instance. The recovery of nucleotides from this type of column has always been 95 per cent or higher. Corrections were made for the nonspecific absorption at 260 mμ by subtracting the E_{260} readings of solutions of appropriate concentrations of formic acid or ammonium formate which had been run through blank resin columns.

The results are reported in terms of micromole quantities of the nucleo-

tides in each 3.0 ml. of incubation medium. These quantities were calculated on the basis of optical density values for solutions containing 1 μmole per ml. of the respective substances (with 1 cm. light path) of 10.0 for the uridine nucleotides and 14.0 for the adenosine nucleotides at 260 mμ in acid solution (pH 1 to 3).

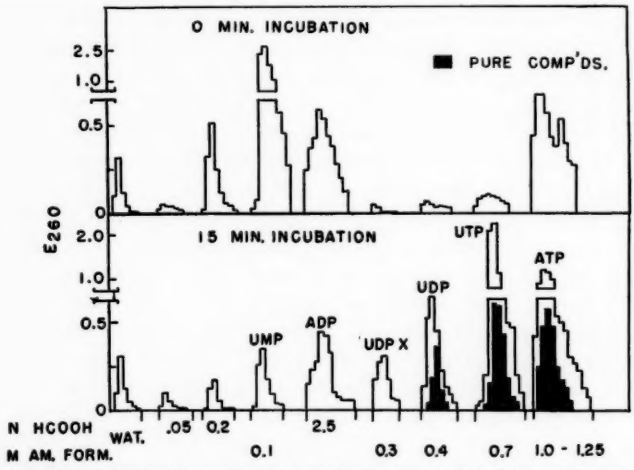


FIG. 1. Chromatographic separation of the products of 5′-uridine monophosphate phosphorylation. Each step in the chromatogram represents a 5 ml. sample eluted from the column. The blackened areas denote the positions of the purified compounds on this combined system. In addition to the standard components, each 3.0 ml. of incubation medium initially contained 160 mg. equivalents of rat liver cytoplasm, 4.5 μmoles of UMP, and 2.5 μmoles of ATP. The initial incubation volume was 7.0 ml. Ordinarily 1.7 N formic acid is used to elute UMP. However, when this is done, IP comes off the column together with ADP (2.5 N formic acid), and when the IP contains P^{32}, as it does in this instance, accurate determinations of the specific activity of IP and ADP are impossible. Hence, 0.1 M ammonium formate was used in this instance, because it elutes IP as well as UMP and prevents contamination of IP^{32} with ADP, which is eluted with 2.5 N formic acid in this case.

Materials—The P^{32} used here was obtained in the carrier-free form from the Oak Ridge National Laboratory of the Atomic Energy Commission.[2] All P^{32} counts were made directly on the samples eluted from the ion exchange columns with a Geiger-Müller dip counter and a Berkeley decimal scaler.[2] Corrections were always made for the decay rate of P^{32}. The specific activities reported here represent averages of P^{32} counts on at least four successive 5 ml. fractions. (The average deviation of the specific

[2] These measurements were made under the direct supervision of Dr. Charles Heidelberger, to whom we acknowledge this valuable help.

activities of samples from the same peak never exceeded ±7 per cent, and the standard deviations never exceeded ±10 per cent.)

The AMP and ATP used were obtained from the Pabst Laboratories, Milwaukee. 5′-UMP and UTP were also supplied by the Pabst Laboratories in both crude and purified form, and UDP was supplied by the same laboratory in a partially purified form. Before use the crude samples were purified by anion exchange chromatography, lyophilization, and barium acetate-alcohol fractionation. The purified UTP samples contained 5 to 10 per cent UDP and a much smaller amount of UMP. There was close correspondence between the uridine moiety of each of the purified samples (as calculated from molar extinction coefficients at 260 mμ in acid solution) and the micromolar quantities of total and labile phosphate present.

Results

Products of 5′-UMP Phosphorylation—Fig. 1 shows that 5′-UMP was almost entirely used up in the course of a 15 minute incubation with the "cytoplasmic fraction" of 160 mg. equivalents of liver cells at 30°.[3] The compounds formed during this time were uridine, a UDPX compound which is probably the UDP glucose-galactose coenzyme of Leloir (5–7), UDP, UTP, and small amounts of two other unidentified compounds not shown in this chromatogram. The products (UDP, UTP, and UDPX) were identified by (1) the position of each relative to that of a highly purified known compound on this combined system as indicated in Fig. 1, (2) the similarity of the ultraviolet absorption spectra in acid and alkali to reference compounds, (3) the close correspondence of the quantity of the uridine moiety in each sample as determined by molar extinction at 260 mμ to the quantities of total and labile phosphate (the ratios of uridine to labile to total phosphate are 1.0:1.0:2.08 for UDP; 1.0:1.95:2.80 for UTP), and, in addition, in the case of the suspected UDPG compound, (4) paper chromatography and sugar tests. In the last instance the results of paper chromatography of the products of mild hydrolysis of a UDPX sample (0.007 N HCl at 100° for 5 minutes) on a butanol-acetic acid system showed that glucose and possibly some galactose are present (4).

Localization of Enzyme Systems That Phosphorylate UMP and UDP—In studying the cellular distribution of the enzyme systems that phosphorylate the uridine nucleotides, it was of particular interest to compare the action of isolated and recombined cell fractions on UMP, on the one hand, with their action on UDP, on the other. The results of two typical ex-

[3] It can also be reported that dihydro UMP, the product of hydrogenation of 5′-UMP, is phosphorylated to di- and triphosphate compounds which appear to be analogous to UDP and UTP. Thus far it has not been possible to phosphorylate 3′-UMP under conditions similar to those of this experiment.

periments illustrating this comparison are presented in Table I and Fig. 2. Table I includes the data for oxygen consumption and changes in the levels of inorganic phosphate during an incubation period of 40 minutes, and Fig. 2 is a graphic representation of the corresponding changes in the nucleotide levels during the same period.

TABLE I

Oxygen Uptake and Orthophosphate Change during UTP and UDP Formation from UMP (Experiment 1) and during UTP Formation from UDP (Experiment 2)

Experiments 1 and 2 correspond to the upper and lower portions of Fig. 2, respectively. The data for changes in IP represent the differences between determinations at zero time and those at the end of the incubation period. Each figure is an average of data from duplicate flasks. The figures for O_2 consumption represent the sum of three separate 10 minute determinations. 160 mg. equivalents of the ice-cold tissue suspensions were added to the cold incubation media in Warburg flasks. After 10 minutes equilibration at 30°, measurements of O_2 consumption were begun. 40 minutes later the flasks were placed in cracked ice, and their contents were immediately deproteinized. In addition to the standard components, the incubation medium initially contained 2.7 μmoles of UMP and 1.35 μmoles of ATP in Experiment 1 and 2.0 μmoles each of UDP and ATP in Experiment 2.

Cell fraction	Experiment 1			Experiment 2		
	O_2 consumed in 30 min.	Change in IP in 40 min.		O_2 consumed in 30 min.	Change in IP in 40 min.	
		Actual	Calculated*		Actual	Calculated*
	μl.	*μmoles*	*μmoles*	*μl.*	*μmoles*	*μmoles*
Mitochondria	28	−2.2	−2.0	20	−3.8	−3.0
Supernatant from mitochondria	22	+4.3	+5.0			
Fluffy layer	26	−2.0	−0.7			
Supernatant + mitochondria	95	−6.7	−3.9			
" + " + fluffy layer	93	−6.7	−3.8	122	−2.5	−2.4
Nuclei + mitochondria	89	−1.0	+0.5	52	−0.3	−0.6
Whole homogenate	255	−3.9	−2.6	205	−2.0	−1.8

* Calculated from the observed changes in the nucleotides.

Reading from left to right, the charts illustrate that the isolated mitochondrial fraction (MIT.) has no action on UMP, while it rapidly phosphorylates UDP to UTP. Accompanying the formation of UTP from UDP is the disappearance of an almost equivalent amount of orthophosphate (Table I, Experiment 2). In contrast to the action of mitochondria, the supernatant liquid from mitochondria (S_2) is able to form UDP from UMP, and, in addition, dephosphorylate UMP to uridine. The fluffy layer (F). that fraction which is swirled off the top of the mitochondria,

acts chiefly to dephosphorylate UMP to uridine. As would be expected, the recombination of the supernatant liquid and mitochondrial fractions results in a vigorous phosphorylation of UMP to UDP and UTP, with the

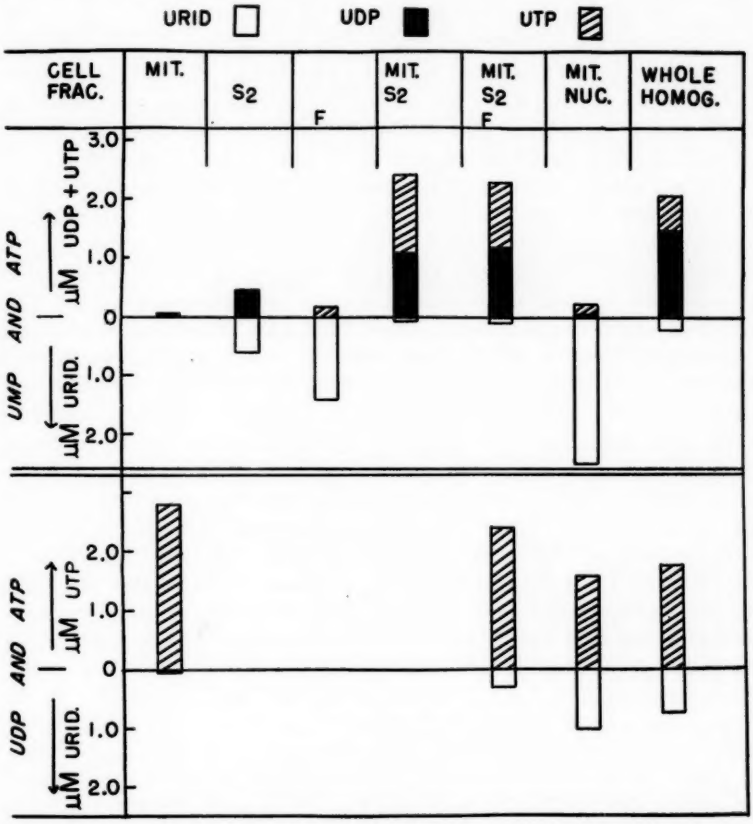


FIG. 2. Comparison of the action of isolated and recombined liver cell fractions on UMP and UDP. The upper chart shows the action of the cell fractions on UMP, and the lower chart the action of the same cell fractions on UDP. The height of the blackened areas above the 0 line in the upper chart represents the amount of UDP formed during the incubation. In both the upper and lower charts the height of the cross-hatched areas represents the extent of UTP formation, while the length of the open areas below the 0 lines represents the amount of uridine formed during the incubation period. The 0 line in the upper chart denotes no action on UMP, and in the lower chart no action on UDP. The symbols MIT., S_2, F, NUC., and URID., refer to mitochondria, supernatant liquid from mitochondria, fluffy layer, nuclear fraction, and uridine, respectively. The conditions of the experiments were the same as those reported in Table I.

disappearance of inorganic phosphate (Table I). The nuclear fraction (NUC. and MIT.) has the greatest capacity of any of the cell fractions under these conditions to dephosphorylate UMP and UDP to uridine, and the presence of nuclei in the whole homogenate reduces the phosphorylating potential of this system slightly, as shown in Fig. 2. These results clearly indicate that UTP is not formed by the transfer of an intact pyrophosphate group to UMP, but rather by a two-stage phosphorylation process involving first the formation of UDP from UMP in the presence of the supernatant liquid from mitochondria (*i.e.*, microsomes and soluble enzymes) and then the formation of UTP from UDP by mitochondria.

Up to this point the discussion has been concerned with the phosphorylation of UMP and UDP in systems carrying on oxidation. It is appropriate now to refer to the results in Table II. Flask 2 illustrates that the phosphorylation of UMP to UDP by the supernatant fraction from mitochondria can proceed in the virtual absence of oxidation, provided ATP has been added to the system. When both ATP and oxidative substrate are omitted, UMP is rapidly dephosphorylated to uridine, accounting for the increase in IP seen in Flask 3 in Table II. It will be shown later (Fig. 5) that the addition of ATP is not necessary for UTP synthesis from UMP when oxidation is taking place. The large increases in IP in Flasks 2 and 5 are due to the breakdown of ATP to AMP.[4] Thus, from the results presented so far, it can be stated that a two-step pathway exists in the cytoplasm of the liver cell for the phosphorylation of UMP to UTP.

Reversibility of Transphosphorylations between Adenosine and Uridine Nucleotides—The results in Fig. 3 are presented in order that the time-course of formation of UDP and UTP from UMP (*A* and *B*) in the presence of added ATP can be compared with the time-course of formation of ADP and ATP from AMP in the presence of added UTP (*C* and *D*).[5] The general similarity of the events in the two cases is evident. In the former instance, as UDP and UTP are formed from UMP, the level of ATP is seen to decrease, resulting in the formation of an equivalent amount of AMP and ADP (*B*). The fact that the actual decrease in the amount of orthophosphate (solid line, upper part of chart *A*) during this time

[4] In another experiment with mitochondria alone, in which oxidation was completely inhibited by an impurity in the added ATP^{32} which had been purified only on a Dowex 50 column, it was found that UTP formation from added UDP proceeded to the extent of 65 per cent of that of the control to which no ATP^{32} had been added. This finding indicates that there is an enzyme in mitochondria which can form UTP from UDP and ATP under non-oxidative conditions.

[5] The curves for UDPX and uridine formation are not shown in Fig. 3, but it can be reported that uridine formation amounts to 5 to 10 per cent of the UMP initially added, while UDPX formation amounts to about 25 to 50 per cent of the UDP generated at 40 minutes by the cytoplasm of liver cells.

corresponds to the theoretical decrease in orthophosphate (dotted line, *A*) which was calculated[6] from the observed changes in the nucleotides indicates that this decrease in ATP is not simply a dephosphorylation, but is the result of a transfer of phosphate from ATP to UMP and UDP. The same general situation holds in the case of ADP and ATP formation from AMP (*C* and *D*). Here, as AMP is removed and ADP and ATP are formed (*C*), there is a corresponding decrease in the level of UTP and the appearance of an equivalent amount of UDP and UMP (*D*). Once again the theoretical decrease in orthophosphate is almost the same as the observed changes (*C*).

TABLE II

Comparison between Action of Oxidative and Non-Oxidative Systems on UMP

Unless otherwise indicated the incubation medium initially contained 160 mg. equivalents of the cell fractions, 4.0 μmoles of UMP, and 5.0 μmoles of ATP. The time of incubation was 40 minutes. The figures for oxygen uptake represent the sum of three 10 minute determinations and those for IP are for the entire incubation period. Otherwise the experimental conditions were the same as those reported in Table I.

Flask No.	Cell fraction	Oxidative substrate	ATP	O_2 uptake in 30 min.	Change in IP in 40 min.	UDP formed in 40 min.	UTP formed in 40 min.
				μl.	*μmoles*	*μmoles*	*μmoles*
1	Supernatant from mitochondria	+	+	52	+1.8	+1.0	0.3
2	" " "	0	+	10	+12.2	+0.8	0
3	" " "	0	0	5	+5.1	0	0
4	" * + "	+	+	140	−1.8	+1.8	+1.2
5	" * + "	0	+	12	+12.9	+0.8	0

* Supernatant from mitochondria.

From these results it can be said that the reactions involved in the formation of UDP and UTP from UMP under the conditions of these studies are about as rapid as those involved in the formation of ADP and ATP from AMP. It is also clear that reversible transphosphorylation reactions are taking place between the uridine and adenosine nucleotides. It is not possible, however, to come to any conclusion at this time about the exact nature of all of the primary products formed or the exact position of the equilibrium because of the complexity of the system used. In this system the position of the equilibrium does not depend solely on the transphosphorylations being studied, but on a number of other reactions as well

[6] This calculation is justified on the basis of the fact that the total extinction at 260 mμ accounted for as either uridine or adenosine nucleotide remained constant during the course of all the experiments reported here.

(29, 30). More information on the question of the reversibility of these reactions is included in another section of the paper.

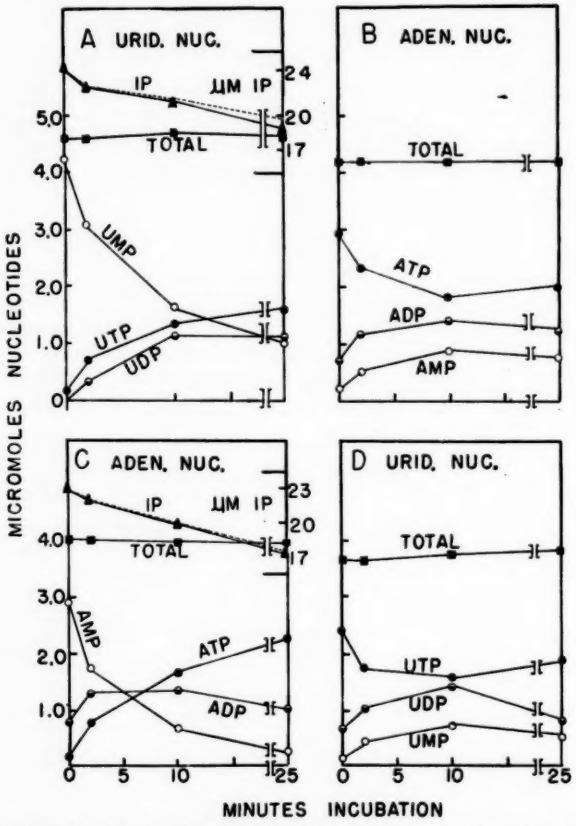


FIG. 3. Reversibility of transphosphorylations between adenosine and uridine nucleotides. The upper charts (*A* and *B*) show the time-course of UMP phosphorylation in the presence of added ATP, and the lower charts (*C* and *D*) the time-course of AMP phosphorylation in the presence of added UTP. In addition to the standard components each 3.0 ml. of incubation medium contained 160 mg. equivalents of cytoplasm, 4.0 μmoles of UMP, and 3.0 μmoles of ATP in Experiment 1 (*A* and *B*) and 3.0 μmoles of AMP and 2.4 μmoles of UTP in Experiment 2 (*C* and *D*). The initial incubation volume was 13.0 ml. in both experiments.

Adenosine Nucleotides As Preferential Phosphate Acceptors; Competition between UMP and AMP—Experiments were set up in order that the rate of phosphorylation of AMP in the presence and absence of UMP could

be compared with the rate of phosphorylation of UMP in the presence and absence of AMP. No ATP was added to these systems in order that the limiting factor in the competitive situation might be the small amount of ATP initially present in the tissue. It was reasoned that, if the adenosine nucleotides were preferential acceptors of phosphate, then in these systems the rate of formation of UTP from UMP would reflect greater competition by AMP than would the rate of ATP formation from AMP reflect competition by UMP. The results are presented in Figs. 4 and 5 so that the proper comparisons can most easily be made.[5] The left-hand section of Fig. 4 illustrates that the rate of disappearance of AMP is only slightly

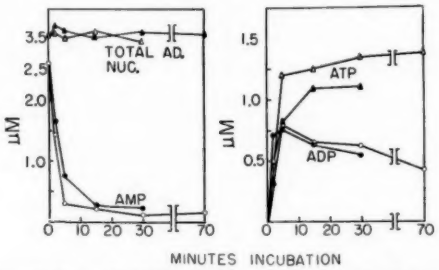


FIG. 4. Phosphorylation of AMP in the presence and absence of UMP. The curves with the open symbols (Experiment 1) represent the changes in the adenosine nucleotides in the absence of added UMP, and those with the closed symbols (Experiment 2) represent the corresponding changes in the adenosine nucleotides in the presence of added UMP. In addition to the standard components each 3.0 ml. of incubation medium initially contained 160 mg. equivalents of cytoplasm and 2.6 μmoles of AMP in Experiments 1 and 2, and, in addition, 2.2 μmoles of UMP in Experiment 2. The initial incubation volume was 13.0 ml. in both experiments.

decreased by the presence of UMP, while the right-hand chart shows that the formation of ATP is reduced about 20 per cent at 5 minutes by the presence of UMP. In contrast to this situation is that illustrated in Fig. 5. Here the rate of disappearance of UMP is seen to be greatly reduced by the presence of AMP (75 per cent at 2 minutes). The right-hand chart shows that the extent of formation of UTP is 55 per cent lower at 5 minutes in the presence of AMP than in its absence, while UDP appears to pile up as a result of this decreased UTP formation.

Since UDP formation from UMP is actually increased in the presence of AMP, it would appear that the adenosine nucleotides exert the competitive effect chiefly at the site of formation of UTP from UDP. Although these results suggest that the adenosine nucleotides are preferentially phosphorylated in a competitive situation with the uridine nucleotides, they do not supply any evidence bearing directly on the question of whether or

not the adenosine nucleotides are actually intermediates between oxidative phosphorylation donors and the uridine nucleotides. These results can be explained in several ways. It is conceivable, for example, that the uridine and adenosine nucleotides could compete for the same enzyme, that they could compete for a limited amount of oxidative phosphorylation donors, or that they could compete for the small amount of ATP in the tissue.

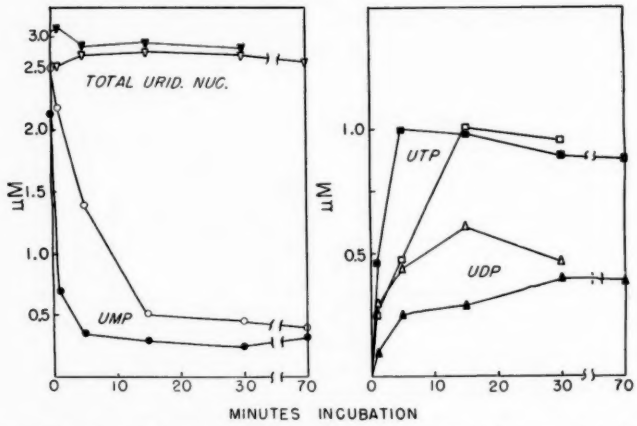


FIG. 5. Phosphorylation of UMP in the presence and absence of AMP. The curves with the closed symbols represent the changes in the uridine nucleotides in the absence of added AMP (Experiment 1), and those with open symbols the corresponding changes in the uridine nucleotides in the presence of added AMP (Experiment 2). In addition to the standard components each 3.0 ml. of incubation medium initially contained 160 mg. equivalents of cytoplasm and 2.5 μmoles of UMP in Experiments 1 and 2 and, in addition, 2.6 μmoles of AMP in Experiment 2.

Studies with P^{32}-Labeled ATP and IP—To answer the question of whether or not the adenosine nucleotides are actually intermediates between oxidative phosphorylation donors and the uridine nucleotides experiments were carried out with P^{32}-labeled ATP in one instance and with P^{32}-labeled orthophosphate in the other. The experiment with P^{32}-labeled ATP was done in the following way. ATP of very high specific activity (280,000 c.p.m. per μmole) was prepared from AMP and IP^{32} by oxidative phosphorylation with mitochondria. The ATP^{32} formed in this way was isolated by chromatographing the acid-soluble extract obtained after 15 minutes incubation on a Dowex 1 (X10) anion exchange column by elution with formic acid. It was purified by lyophilization and barium acetate-alcohol fractionation. Then it was possible to set up a system which con-

tained UMP, a large pool of non-radioactive IP, oxidative substrate, and only 0.07 μmole of added ATP^{32}. It had been determined previously that the addition of this amount of ATP^{32} to the medium would not appreciably change the concentration of ATP initially present in the tissue, while it would permit accurate radioactive counts to be made. In this way it proved possible to study the pathway of UDP and UTP formation that predominates under conditions comparable to those in Fig. 4 in the absence of added ATP. The specific activities and quantities of all of the mono-, di-, and triphosphates and IP were determined at 0, 2, 10, and 25

TABLE III

Rate of Incorporation of P^{32} from ATP^{32} into UDP and UTP during Their Formation from UMP

In addition to the standard components each 3.0 ml. of incubation medium initially contained 160 mg. equivalents of cytoplasm, 3.7 μmoles of UMP, and 0.07 μmole of ATP^{32}. The initial incubation volume was 13.0 ml. Specific activity in counts per minute per micromole.

Incubation time, *min.* . .	0		2		10		25	
	Specific activity	μmoles	Specific activity	μmoles	Specific activity	μmoles	Specific activity	μmoles
ATP	38,000	0.2*	4,200	0.3	2,300	0.3	2,000	0.3
UTP		0	10,500	0.3	2,500	0.9	1,800	0.9
ADP	16,000	0.5	1,500	0.4	900	0.4		
UDP		0.1	7,400	0.5	1,400	0.9	840	0.6
IP	175	33.0	375	31.0	510	29.0	610	28.4
Total c.p.m.	21,375		19,700		19,560		20,410	

* Includes 0.07 μmole of added ATP^{32}.

minutes, and the results are shown in Table III. It can be seen that the specific activity of the orthophosphate which is one-hundredth that of ATP initially is one-twelfth that of ATP, one-twentieth that of UDP, and one-thirtieth that of UTP at 2 minutes. This situation can be explained by assuming that UDP and UTP are formed by the transfer of phosphate from ATP or ADP to UMP and UDP and that at the same time the labile phosphate groups of ATP are turning over more rapidly with the large relatively non-radioactive orthophosphate pool than with the labile phosphate groups of UTP. The specific activities of the monophosphates were negligible in all these experiments.

This experiment indicates that the predominant pathway for the formation of UDP and UTP from UMP is the transfer of phosphate from the adenosine nucleotides, bearing out the idea that the latter are preferential

intermediates between oxidative phosphorylation donors and the uridine nucleotides. It should also be pointed out that the specific activity of the terminal phosphate of UTP, which can be calculated by subtracting the specific activity of UDP from that of UTP,[7] is higher than that of the second phosphate.

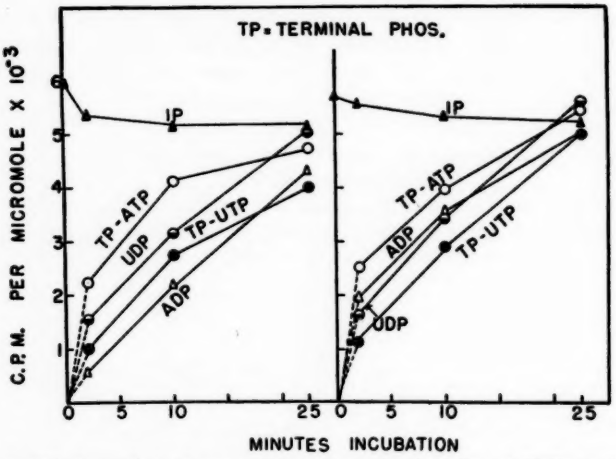


FIG. 6. Dilution effect of non-radioactive pools of ATP and UTP on the incorporation of IP^{32} into the di- and triphosphates of uridine and adenosine during their formation from UMP and AMP, respectively. The left-hand chart shows the effect of a non-radioactive pool of ATP on the incorporation of IP^{32} into UDP and UTP during their formation from UMP (Experiment 1), and the right-hand chart the effect of a non-radioactive pool of UTP on the incorporation of IP^{32} into ADP and ATP during their formation from the AMP (Experiment 2). In addition to the standard components and 160 mg. equivalents of cytoplasm each 3.0 ml. of incubation medium initially contained 24 μmoles of IP with 141,000 c.p.m., 4.3 μmoles of UMP, and 2.9 μmoles of ATP in Experiment 1, and 23 μmoles of IP with a total of 128,000 c.p.m., 3.0 μmoles of AMP, and 2.7 μmoles of UTP in Experiment 2. The initial incubation volume was 13 ml. in both experiments.

In the second approach to this problem the effect of a non-radioactive pool of ATP on the incorporation of IP^{32} into UDP and UTP during their formation from UMP has been compared with the effect of a non-radioactive pool of UTP on the incorporation of IP^{32} into ADP and ATP during

[7] It can be reported that in other experiments similar to the one described above there is very close agreement between the specific activities of the terminal phosphates of UTP and ATP calculated in this way and those actually measured by determining the specific activities of ADP and UDP resulting from partial hydrolysis of the UTP and ATP samples.

their formation from AMP. In each of these experiments the extent to which newly incorporated IP^{32} is diluted by the non-radioactive triphosphate pool is a measure of the rate of the transphosphorylation reactions taking place and is, therefore, an indication of the predominant pathway of phosphorylation in each instance. In Fig. 6 the specific activities of terminal phosphates of ATP and UTP calculated by subtracting the specific activities of their respective diphosphates[7] are plotted along with those of the diphosphates and IP. The left-hand section of Fig. 6 shows that during the formation of UDP and UTP from UMP the specific activity of the terminal phosphate of ATP is considerably higher at 2 and 10 minutes than that of the terminal phosphate of UTP or UDP.[8] Since the specific activity of the IP^{32} is much higher than any other phosphate group at 2 and 10 minutes, this finding would indicate that the IP^{32} is incorporated into the terminal phosphate of ATP before forming UDP and UTP. The right-hand part of Fig. 6 shows that during the formation of ADP and ATP from AMP the specific activity of the terminal phosphate of ATP is higher than that of UTP and UDP.[8] With like reasoning this would indicate that in this system the primary pathway for the incorporation of IP^{32} into newly formed ADP and ATP under these experimental conditions is directly from oxidative phosphorylation donors and not from UTP or UDP. These results also show that the transphosphorylation reactions are reversible, a conclusion already reached on the basis of other evidence presented earlier.

DISCUSSION

Several points regarding the intracellular distribution of the enzyme systems that phosphorylate and dephosphorylate the uridine nucleotides are worthy of further discussion. The first is that, while mitochondria can form ATP from either AMP or ADP (29, 30), they can form UTP only from UDP and have to be supplemented with the supernatant fraction (S_2) to generate UTP from UMP. The second point is that the dephosphorylation of 5′-UMP like that of 5′-AMP (31) is brought about chiefly by the nuclear fraction, although the fluffy layer is quite active on 5′-UMP in this respect also.

Other results presented here may have some bearing on the composition of the fluffy layer, about which there has been some discussion (32–34). The results of a recent study by Laird *et al.* (33) indicate that this layer has biochemical properties which differentiate it from both mitochondria and the supernatant liquid from mitochondria, and these authors suggest

[8] There is a significant difference between the specific activity of the terminal phosphates of ATP and those of UTP and UDP at 2 and 10 minutes ($P < 0.02$ in each instance).

that this layer might be unique as a cell fraction. Results presented here are in agreement with this idea, since they show that the fluffy layer has a much greater capacity (at least 2-fold) to dephosphorylate 5′-UMP than either the mitochondria or the supernatant liquid from mitochondria.

It has been shown that the cytoplasm catalyzes a rapid and reversible transphosphorylation between the uridine and the adenosine nucleotides. It has also been noted that the formation of both UDP and UTP can occur to a lesser extent in the absence of oxidation provided ATP is added to the system. When ATP is not added and in addition oxidation is prevented, the formation of UDP and UTP no longer takes place. Transphosphorylations of a similar nature have been observed by Krebs and Hems (35) between the adenosine and inosine nucleotides with pigeon breast muscle and by Berg and Joklik (15, 16) between UDP and ATP in yeast and rabbit muscle extracts. It would appear that transphosphorylations of the type studied here are general to many classes of nucleotides in view of recent observations that IP^{32} equilibrates very rapidly with the di- and triphosphate pools of adenosine, uridine, guanosine, and cytidine in rat liver *in vivo* (1). Preliminary studies with unfractionated rat liver cytoplasm have shown that cytidine monophosphate and deoxycytidine monophosphate are rapidly converted to the corresponding di- and triphosphates under conditions similar to those of the present work (36).

Although experiments with IP^{32} and ATP^{32} indicate that the adenosine nucleotides are preferential intermediates between oxidative phosphorylation donors and the uridine nucleotides under the experimental conditions observed here, they do not answer the question of whether or not the adenosine nucleotides are obligatory intermediates. To get further information on this question it would seem necessary to use a system that had been reconstituted from purified or at least partially purified enzymes, as Sanadi and Ayengar (37) have done in demonstrating that guanosine diphosphate is required for ATP synthesis from succinyl coenzyme A, ADP, and orthophosphate during diphosphopyridine nucleotide reduction with α-ketoglutaric dehydrogenase, and as Lieberman *et al.* (38) and Strominger *et al.* (39) have done with uridine nucleotides, using liver extracts.

It is believed that when the pattern of phosphorylation of all of the 5′-monophosphates is established it may enable us to see how the reactions of oxidative phosphorylation in the intact liver mitochondria can be integrated with nucleic acid synthesis (40), which apparently takes place in another part of the cell.

The authors would like to acknowledge the valuable technical assistance of Mrs. Heidi Haeberli who helped with the Beckman readings and P^{32}

counting. The authors are indebted to the Pabst Laboratories, Milwaukee, for supplying them with uridine compounds (5′-UMP, dihydro UMP, UDP, and UTP) at a time when they were not available commercially.

SUMMARY

An anion exchange column method has been described for the separation of the uridine and adenosine nucleotides. It has been shown by this method that the "cytoplasmic fraction" of rat liver cells can phosphorylate 5′-UMP to UDP, UTP, UDPG, and two other unidentified compounds during oxidative phosphorylation. The isolated mitochondrial fraction is able to phosphorylate UDP to UTP, but has no action on UMP. The supernatant liquid from the mitochondria is able to phosphorylate UMP to UDP and some UTP, while the chief action of the nuclei appears to be the dephosphorylation of UMP. It has been shown that there are rapid and reversible transphosphorylation reactions between the uridine and adenosine nucleotides. With use of ATP^{32} and IP^{32} it has been possible to demonstrate that the formation of UDP and UTP from UMP during oxidative phosphorylation proceeds predominantly by the transfer of phosphate from the terminal phosphate of ATP to UMP and UDP. It has been concluded that under the present experimental conditions the adenosine nucleotides behave as preferential intermediates between the uridine nucleotides and oxidative phosphorylation donors.

BIBLIOGRAPHY

1. Potter, V. R., Herbert, E., Takagi, Y., Siekevitz, P., and Brumm, A., *Federation Proc.*, **13**, 276 (1954).
2. Schmitz, H., Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **209**, 41 (1954).
3. Schmitz, H., Potter, V. R., Hurlbert, R. B., and White, D. M., *Cancer Res.*, **14**, 66 (1954).
4. Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **209**, 1 (1954).
5. Cardini, C. E., Paladini, A. C., Caputto, R., and Leloir, L. F., *Nature*, **165**, 191 (1950).
6. Caputto, R., Leloir, L. F., Cardini, C. E., and Paladini, A. C., *J. Biol. Chem.*, **184**, 333 (1950).
7. Leloir, L. F., *Advances in Enzymol.*, **14**, 196 (1953).
8. Park, J. T., *J. Biol. Chem.*, **194**, 877 (1952).
9. Park, J. T., *J. Biol. Chem.*, **194**, 885 (1952).
10. Park, J. T., *J. Biol. Chem.*, **194**, 897 (1952).
11. Kornberg, A., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1**, 411 (1951).
12. Kalckar, H. M., *Biochim. et biophys. acta*, **12**, 250 (1953).
13. Munch-Peterson, A., Kalckar, H. M., Cutolo, E., and Smith, E. E. B., *Nature*, **172**, 1036 (1953).
14. Kalckar, H. M., Braganca, B., and Munch-Peterson, A., *Nature*, **172**, 1038 (1953).
15. Berg, P., and Joklik, W. K., *Nature*, **172**, 1008 (1953).

16. Berg, P., and Joklik, W. K., *Federation Proc.*, **13,** 182 (1954).
17. Leloir, L. F., and Cabib, E., *J. Am. Chem. Soc.*, **75,** 5445 (1953).
18. Leloir, L. F., and Cardini, C. E., *J. Am. Chem. Soc.*, **75,** 6084 (1953).
19. Tomayo, M. L., and Alvarez, E. F., *Nature*, **173,** 584 (1954).
20. Smith, E. E. B., and Mills, G. T., *Biochim. et biophys. acta*, **13,** 386 (1954).
21. Smith, E. E. B., and Mills, G. T., *Biochim. et biophys. acta*, **13,** 587 (1954).
22. Kalckar, H. M., *Science*, **119,** 479 (1954).
23. Ling, K.-H., and Lardy, H. A., *J. Am. Chem. Soc.*, **76,** 2842 (1954).
24. Dutton, G. J., and Storey, I. D. E., *Biochem. J.*, **48,** p. xxix (1951).
25. Dutton, G. J., and Storey, I. D. E., *Biochem. J.*, **53,** p. xxxvii (1953).
26. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183,** 123 (1950).
27. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66,** 375 (1925).
28. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., *J. Biol. Chem.*, **209,** 23 (1954).
29. Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, **191,** 485 (1951).
30. Barkulis, S. S., and Lehninger, A. L., *J. Biol. Chem.*, **190,** 339 (1951).
31. Novikoff, A. B., Podber, E., Ryan, J., and Noe, E., *J. Histochem. and Cytochem.*, **1,** 27 (1953).
32. Schneider, W. C., and Hogeboom, G. H., *Cancer Res.*, **11,** 1 (1951).
33. Laird, A. L., Nygaard, O., Ris, H., and Barton, A. D., *Exp. Cell Res.*, **5,** 147 (1953).
34. Muntwyler, E., Seifter, S., and Harkness, D. M., *J. Biol. Chem.*, **184,** 181 (1950).
35. Krebs, H. A., and Hems, R., *Biochim. et biophys. acta*, **12,** 172 (1953).
36. Hecht, L. I., Potter, V. R., and Herbert, E., *Biochim. et biophys. acta*, **15,** 134 (1954).
37. Sanadi, D. R., and Ayengar, P., *Federation Proc.*, **13,** 287 (1954).
38. Lieberman, I., Kornberg, A., and Simms, E. S., *J. Am. Chem. Soc.*, **76,** 3608 (1954).
39. Strominger, J. L., Heppel, L. A., and Maxwell, E. S., *Arch. Biochem. and Biophys.*, **52,** 488 (1954).
40. Potter, V. R., in Begg, R. W., Canadian cancer conference, in press.

FAT METABOLISM IN HIGHER PLANTS

IV. PREPARATION OF SOLUBLE FATTY ACID OXIDASES FROM PEANUT MICROSOMES

By T. E. HUMPHREYS and P. K. STUMPF*

(From the Department of Plant Biochemistry, University of California, Berkeley, California)

(Received for publication, August 16, 1954)

In a previous paper from this laboratory (1) it was reported that the microsomal particles from the cotyledons of germinated peanuts catalyzed the oxidation of the higher fatty acids. With this microsomal system no cofactor requirements were demonstrable for $C^{14}O_2$ production from palmitate-1-C^{14}, while DPN[1] was specifically required for $C^{14}O_2$ production from internally labeled palmitate. Synthetic palmityl-CoA was found to be inactive, and evidence was presented to indicate that the tricarboxylic acid cycle is not responsible for CO_2 production from palmitate oxidation as catalyzed by the peanut microsomes.

By treating the peanut microsomes with sodium choleate, the fatty acid-oxidizing enzymes have been rendered soluble. This paper will report the results of a study on the oxidation of palmitic acid by these soluble enzymes.

EXPERIMENTAL

Reagents—We are deeply indebted to Professor I. L. Chaikoff, Department of Physiology, University of California, for generous samples of palmitic acid-1-C^{14}, -2-C^{14}, -3-C^{14}, -11-C^{14}, and -15-C^{14}.

0.001 M palmitic acid. Sufficient radioactive palmitic acid (approximately 10^5 c.p.m.) was added to carrier palmitic acid to make a total of 2.56 mg. of substrate which was suspended in 5 ml. of distilled water. The suspension was heated to boiling and 1 M NH_4OH was added dropwise until the fatty acid was dissolved. The solution was diluted to a final volume of 10 ml. and stored at −5°. The palmitate solution was always heated to 100° to bring the substrate into complete solution and then added to the Warburg flasks in required amounts.

0.1 per cent DPN "90," Sigma.

* Supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[1] The following contractions are used: DPN, diphosphopyridine nucleotide; CoA, coenzyme A; ATP, adenosinetriphosphate.

0.06 M Veronal-acetate buffer adjusted to the desired pH with 0.1 N HCl.

20 per cent KOH. KOH is dissolved in boiled distilled water.

20 per cent barium acetate. The salt is dissolved in boiled distilled water

10 N sulfuric acid.

Reaction Mixtures—Each Warburg vessel contained 0.1 ml. of enzyme, 0.1 μmole of radioactive palmitate, 0.5 ml. of 0.06 M Veronal-acetate buffer (pH 6.0 when palmitate-1-C^{14} was used as a substrate and pH 6.5 when internally labeled palmitate was used as a substrate), 0.2 ml. of 20 per cent KOH in the center well, 0.3 ml. of 10 N sulfuric acid in the side arm, cofactors as indicated later, and water to a final volume of 2.0 ml. Incubation was for 1 hour at 30°. At the end of the incubation period, acid was tipped from the side arm into the main compartment to stop the reaction and release bound CO_2. Shaking was continued for 10 minutes, after which the contents of the center well were removed with washing, and the respiratory CO_2 was precipitated as $BaCO_3$ with 20 per cent barium acetate. The precipitate was washed three times with 15 ml. of 50 per cent ethanol, then plated on aluminum disks and counted with a thin window Geiger-Müller tube.

Preparation of Enzymes—The microsomal material used in these experiments was prepared from the cotyledons of germinated peanuts (*Arachis hypogea* L., var. Virginia Jumbo). The procedure for the preparation of the microsomes and the criteria used in designating this particulate fraction as a microsomal one are outlined in a previous communication (1).

Fatty acid-oxidizing enzymes from the microsomes were rendered soluble by the use of sodium choleate. The microsomes were suspended in a small volume of cold 0.2 M KCl, and sodium choleate (10 mg. per ml. of suspension) was added. The choleate-treated suspension was placed in an ice bath for 1 hour and then centrifuged for 15 minutes at 100,000 × *g*. The translucent precipitate and the clear, pale yellow, supernatant fraction from this centrifugation were tested for their ability to catalyze the production of $C^{14}O_2$ from palmitate-1-C^{14} with the results shown in Table I. It is evident from these results that on choleate treatment the fatty acid-oxidizing enzymes become soluble.

The supernatant fraction of the choleate-treated microsomes was made 80 per cent saturated with respect to ammonium sulfate and was centrifuged at 13,000 × *g* for 15 minutes at 4°. The fatty acid-oxidizing enzymes which floated to the top of the centrifuged solution were lifted from the surface of the liquid with a stirring rod and were dissolved in a small volume of cold 0.2 M KCl. This enzyme solution was used to obtain the results reported in this paper and will be referred to as the AS enzyme. The microsomes and the fatty acid-oxidizing enzymes rendered soluble

could be stored for weeks at −5° with no noticeable loss of activity. The AS enzyme, however, lost 50 to 75 per cent of its activity on standing overnight at −5°. The AS enzyme, therefore, was freshly prepared from the supernatant fraction of the choleate-treated microsomes before each experiment.

Results

Components of System—The supernatant fraction obtained with no further treatment from the choleate-treated microsomes oxidized palmitic acid at a rate that was somewhat higher than that of the microsomes from which it was prepared. The component requirements were the same; no added cofactors were necessary for $C^{14}O_2$ production from palmitate-1-C^{14}, while DPN was necessary for $C^{14}O_2$ production from internally labeled

TABLE I

Distribution of Enzyme Activity in Various Preparations

Enzyme system	Per cent of total C^{14} as $C^{14}O_2$
Microsomes	18
" + choleate	27
Supernatant fraction from centrifuged choleate-treated microsomes	33
Sediment from centrifuged choleate-treated microsomes	7
Supernatant fraction + sediment	27

palmitate. Dialysis of the supernatant fraction against 15 times its volume of 0.2 M KCl for 24 hours caused no decrease in enzyme activity.

However, the enzyme prepared by fractionation of the supernatant solution with ammonium sulfate had low activity towards palmitic acid-1-C^{14}. Full activity could be restored upon the addition of DPN. Thus, in a typical experiment, the following percentage conversions of added C^{14} (as palmitic acid-1-C^{14}) to $C^{14}O_2$ were obtained: supernatant fraction, 60 per cent; AS enzyme, 11 per cent; AS enzyme + DPN, 70 per cent. In all experiments in which DPN served as a cofactor, 0.136 μmole of DPN was added to the Warburg vessels. This amount represented an excess, since, from the results of a DPN concentration experiment, maximal activity was obtained when 0.034 μmole of DPN was added. Higher concentrations of DPN neither increased nor decreased the activity of the AS enzyme.

A substance (or substances), other than DPN, has been found in boiled extracts of peanut cotyledons which stimulates the activity of the AS enzyme. This substance has been partially characterized (2). It is stable

to 0.1 N alkali and 0.1 N acid at 100°, is not adsorbed by charcoal or Dowex 50, but is adsorbed by Dowex 1, and is extractable into ether under acid conditions. The unknown substance present in the boiled extracts of peanut cotyledons is designated as US. Although these properties are suggestive of a short chain organic acid, the only organic acids found in the partially purified peanut extract are glycolic and lactic acids. These two acids were found to activate the AS enzyme to only a slight extent. Acids of the citric acid cycle have also been tested and found to be inert.

US alone has no effect on the production of $C^{14}O_2$ from palmitic acid-2-C^{14}, but, when added along with DPN, the amount of $C^{14}O_2$ produced is more than double that obtained when DPN alone serves as a cofactor. The activity of the AS enzyme towards palmitic acid-1-C^{14}, -2-C^{14}, -3-C^{14}, -11-C^{14}, and -15-C^{14} under various conditions is shown in Table II. The production of $C^{14}O_2$ from palmitic acid-1-C^{14} is not increased by the addition of US when DPN is also present. $C^{14}O_2$ production from palmitic acid-2-C^{14} and palmitic acid-3-C^{14} is markedly increased, however, by the addition of US when DPN is present. It should be emphasized that in the absence of DPN no $C^{14}O_2$ was obtained from internally labeled palmitic acid even though US was present. In addition, a long list of known cofactors and acids of the citric acid cycle were tested as replacements for US, but all were found inert.

TABLE II

Effect of DPN and DPN + US on Oxidation of Palmitic Acid by Fatty Acid-Oxidizing Enzymes (AS Enzyme)

Substrate	Per cent of total C^{14} appearing as $C^{14}O_2$			
	None	US	DPN	DPN + US
Palmitic acid-1-C^{14}	4, 3	13, 31	62, 55	65, 59
" acid-2-C^{14}	0, 0	0, 0	15, 16	41, 32
" acid-3-C^{14}	0, 0	0, 0	2, 1.5	14, 10
" acid-11-C^{14}			1, 1	1, 1
" acid-15-C^{14}			0, 0	0, 0

In a previous paper (1) it was reported that although the microsomal particles from peanut cotyledons catalyze the oxidation of the higher fatty acids the mechanism of this oxidation appears to be quite different from that of the animal system. This conclusion was based, in part, on the fact that, while ATP, CoA, Mg^{++}, and DPN are essential cofactors for the animal fatty acid oxidase system (3), only DPN is required for the internal oxidation of palmitic acid by the peanut microsome. The possi-

bility remained, however, that the intact particles contained sufficient amounts of the other cofactors. If this is so, then the cofactors must be tightly bound to the enzyme, since disrupting the particles with sodium choleate followed by dialysis or by ammonium sulfate fractionation of the resulting enzyme solution, rendered soluble, resulted in an enzyme solution (AS enzyme) that still required only DPN for activity. To remove any tightly bound cofactors that might be present the AS enzyme was treated with "Darco" charcoal and with Dowex 1 resin. Charcoal (10 mg. per

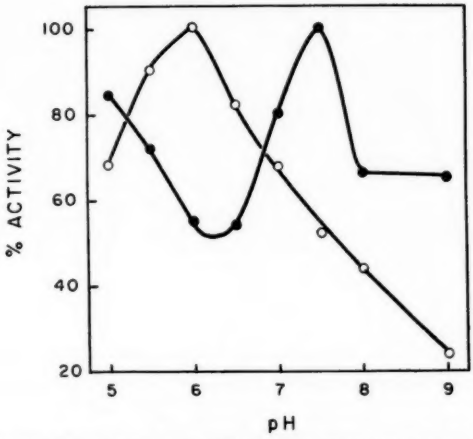


FIG. 1. Effect of pH on $C^{14}O_2$ production from palmitate-1-C^{14} catalyzed by the AS enzyme when either US or DPN was added. 0.06 M Veronal-acetate buffer was used over the entire pH range investigated. The reaction was run for 1 hour at 30°. The per cent activity was calculated by assigning the activities at pH 6.0 and at pH 7.5 as 100 for the DPN and US reactions, respectively. Curve 1 (○), DPN added; Curve 2 (●), US added.

ml.) was added to the enzyme solution, and the resulting suspension, after remaining in an ice bath for 1 hour, was centrifuged and filtered to remove the charcoal. This treatment was repeated three times. The Dowex 1-treated enzyme was prepared by adding a Dowex 1 Cl^- slurry (taken to pH 8 with tris(hydroxymethyl)aminomethane buffer) to the enzyme solution (volume for volume) and, after stirring for 5 minutes in the cold, centrifuging to remove the resin. This treatment was repeated two times. Neither treatment appreciably decreased the activity of the enzyme. In the presence of DPN the addition of CoA, ATP, cocarboxylase, flavin adenine dinucleotide, glutathione, and Fe^{++}, either singly or in various combinations, did not increase the activity of the AS enzyme system over

that obtained when only DPN was added. ATP consistently inhibited to a slight extent, and Fe^{++} was strongly inhibitory.

Effect of pH on Activity—Fig. 1 illustrates the effect of pH on the activity of the AS enzyme towards palmitic acid-1-C^{14} when either DPN or US is present. The pH optimum for the production of $C^{14}O_2$ from palmitic acid-1-C^{14} when DPN serves as a cofactor is 6.0. With US the pH optimum is shifted to 7.5. Although both DPN and US activate the AS enzyme towards palmitic acid-1-C^{14}, the mechanism of the activation, as

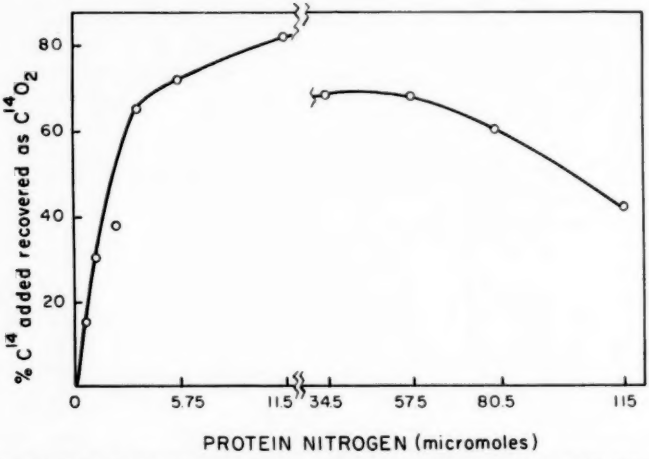


FIG. 2. Effect of AS enzyme content on $C^{14}O_2$ production from palmitate-1-C^{14}. The reaction was run for 1 hour at 30°, and DPN was used to activate the enzyme.

judged from the pH-activity curves, is different. Veronal-acetate buffers (0.06 M) were used over the entire pH range studied.

Effect of Enzyme Concentration—Fig. 2 shows the effect of AS enzyme content on the amount of added C^{14} (as palmitic acid-1-C^{14}) converted to $C^{14}O_2$ in 1 hour. The amount of enzyme is expressed as micromoles of protein nitrogen, and the AS enzyme was activated with an excess of DPN. The curve is linear only at rather low enzyme concentrations, and, instead of forming a plateau, it shows a maximum. When 115 μmoles of protein nitrogen were added, only one-half as much $C^{14}O_2$ was produced as when 11.5 μmoles (optimal amount) were added. The presence of a maximum in the enzyme concentration curve is most probably related to the adsorption of the palmitic acid on the inactive protein present in the AS enzyme solution. Such an adsorption would result in a removal of part of the

substrate from the reaction and would contribute to the variability in the apparent activities of the different enzyme preparations.

Effect of Time on Extent of Oxidation—In Fig. 3 the percentage conversions of added C^{14} (as palmitic acid-1-C^{14}) to $C^{14}O_2$ are plotted as a function of time when the AS enzyme and DPN are added at zero time. In Fig. 4 similar plots are shown with palmitic acid-2-C^{14} and palmitic acid-3-C^{14} as

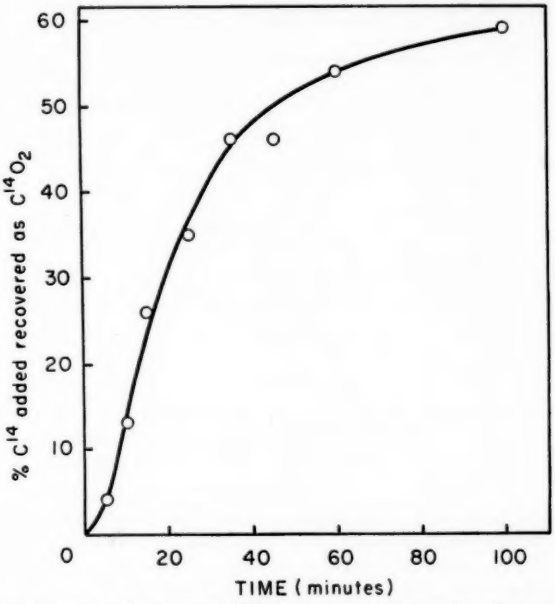


FIG. 3. $C^{14}O_2$ production from palmitate-1-C^{14} catalyzed by the AS enzyme as a function of time. Temperature 30°; pH 6.0.

substrates. The effect of added US in these systems is also recorded. In obtaining these data only 0.1 μmole of palmitic acid was added, since larger amounts were inhibitory.

It can be seen (Fig. 3) that the production of $C^{14}O_2$ from palmitic acid-1-C^{14} is essentially complete at the end of 60 minutes, even though only 55 per cent of the substrate has been oxidized. The percentage of the 1st carbon oxidized to CO_2 varies with the enzyme preparation; preparations releasing over 90 per cent of the 1st carbon as CO_2 have been obtained. Regardless of the apparent activity of the enzyme, however, the reaction is essentially completed at the end of 60 minutes. This is due most prob-

ably not only to the variability in enzyme activity but also to the removal of substrate by adsorption on inactive protein, the amount of which varies with different enzyme preparations.

The time curves for palmitic acid-2-C^{14} and palmitic acid-3-C^{14} show lag periods in which no $C^{14}O_2$ is produced. The lag period is longer for palmitic acid-3-C^{14} than for palmitic acid-2-C^{14}. The addition of US decreases the lag period and also increases the rate of $C^{14}O_2$ production from these internally labeled palmitic acids.

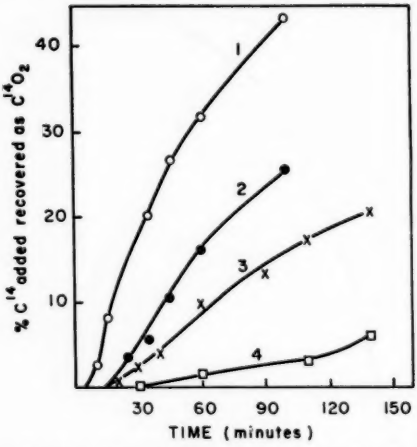


FIG. 4. $C^{14}O_2$ production from palmitate-2-C^{14} and from palmitate-3-C^{14} catalyzed by the AS enzyme as a function of time when either DPN or DPN plus US was added. Temperature 30°; pH 6.5. Curve 1, palmitate-2-C^{14}, DPN + US; Curve 2, palmitate-2-C^{14}, DPN. Curve 3, palmitate-3-C^{14}, DPN + US; Curve 4, palmitate-3-C^{14}, DPN.

When palmitic acid-11-C^{14} was used, no more than 1.5 per cent of the C^{14} was converted to $C^{14}O_2$ even after 4 hours. No $C^{14}O_2$ was produced from palmitic acid-15-C^{14} after 5 hours.

DISCUSSION

As yet, no data are available which would throw light on the nature of the reaction products. The unique difference in cofactor requirements between the microsomal system of peanuts and the fatty acid oxidation systems of animal and bacterial tissues (3, 4) would imply a completely different series of reaction steps. It is hoped that, as analytical techniques for the chromatography of micromolar quantities of fatty acids are developed, the identification of the reaction products, which are undoubtedly

accumulating, will rapidly take place. In addition, the elucidation of the nature of US which appears to participate in the internal oxidation of palmitic acid will be necessary before a detailed explanation is possible.

SUMMARY

The fatty acid-oxidizing enzymes from peanut cotyledon microsomes have been rendered soluble by the use of sodium choleate. The soluble enzymes had the same cofactor requirements as the microsomal enzymes; *i.e.*, no cofactor additions were necessary for $C^{14}O_2$ production from palmitate-1-C^{14}, while the addition of DPN was necessary for $C^{14}O_2$ production from internally labeled palmitic acid. Ammonium sulfate fractionation of the enzymes rendered soluble resulted in an enzyme preparation which required DPN for $C^{14}O_2$ production from both palmitic acid-1-C^{14} and internally labeled palmitic acid.

An unknown substance (or substances) from boiled extracts of peanut cotyledons was found which activated the enzyme towards palmitic acid-1-C^{14} in the absence of DPN and which increased the rate of $C^{14}O_2$ production from internally labeled palmitic acid in the presence of DPN. No other cofactor requirement could be demonstrated.

BIBLIOGRAPHY

1. Humphreys, T. E., Newcomb, E. H., Bokman, A. H., and Stumpf, P. K., *J. Biol. Chem.*, **210**, 941 (1954).
2. Castelfranco, P. A., Stumpf, P. K., Contopoulou, R., and Humphreys, T. E., *Federation Proc.*, **13**, 189 (1954).
3. Mahler, H. R., *Federation Proc.*, **12**, 694 (1953).
4. Barker, H. A., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1**, 204 (1951).

AN OXIDASE FOR REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE*

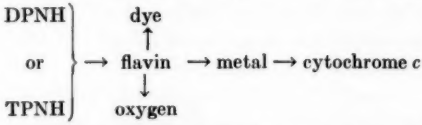
By F. M. HUENNEKENS, R. E. BASFORD, and BEVERLY WESCOTT GABRIO

With the Technical Assistance of S. Felton and E. Nurk

(From the Departments of Biochemistry and Medicine, University of Washington, Seattle, Washington)

(Received for publication, July 14, 1954)

The oxidation of reduced pyridine nucleotides, DPNH[1] and TPNH, has been shown to be mediated by a variety of enzyme systems, some of which have been made soluble and extensively purified, while others have been studied only as particulate preparations. By means of the following schematic diagram, in which the arrows indicate the pathway of electron transport, these enzymes may be classified operationally as cytochrome *c* reductases, diaphorases, or oxidases, depending upon whether the terminal electron acceptor is cytochrome *c*, methylene blue, or oxygen.



In the event that the dye is autoxidizable, a diaphorase then becomes an oxidase and the dye serves as an electron carrier.

The present communication describes the properties of a DPNH oxidase (or diaphorase), purified from pig heart. In the presence of methylene blue or other carriers, this enzyme catalyzes the oxidation of DPNH by oxygen. Certain properties of the enzyme suggest that it is not identical with Straub's diaphorase (2) or cytochrome *c* reductase (3, 4) (which also exhibits diaphorase activity), although both of these enzymes are present

* This work has been supported by funds from Initiative 171, State of Washington, Eli Lilly and Company, the National Institutes of Health, United States Public Health Service, and the Department of the Army, Office of the Surgeon General. A preliminary report of this work was presented at the Forty-fifth meeting of the American Society of Biological Chemists at Atlantic City, April, 1954 (1).

[1] The following abbreviations are used: KG, α-ketoglutaric acid; KGD, α-ketoglutaric dehydrogenase; CoA, coenzyme A; DPN, TPN, di- and triphosphopyridine nucleotides; DPNH, TPNH, reduced DPN and TPN; AMP, ADP, and ATP, adenosinemono-, di-, and triphosphate; ITP, inosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; PCMB, *p*-chloromercuribenzoate; BAL, 2,3-dimercaptol propanol; FMN, riboflavin-5′-phosphate; 4,5-FMN (cyc.), riboflavin-4′,5′-phosphate (cyclic); FAD, flavin adenine dinucleotide; FAD-X, cyclic ester of FAD.

in the same tissue. Because the enzyme is found in preparations of α-ketoglutaric dehydrogenase, a multifunctional enzyme whose molecular weight is about 2×10^6 (5), the possibility has been investigated that the DPNH oxidase is associated with the larger complex.

EXPERIMENTAL

Materials—Chemicals were obtained from the following sources: DPN, DPNH, TPNH, AMP, ADP, ATP, ITP, cytochrome *c*, and PCMB, Sigma; methylene blue and 2,6-dichlorophenolindophenol, Eastman; gramicidin, BAL, acetyl phosphate, and crystalline alcohol dehydrogenase, Mann Research Laboratories; crystalline catalase, Worthington Biochemical Corporation; FMN, Hoffmann-La Roche; AMP (*a*, *b*, and 5′ isomers), adenine, and adenosine, Schwarz Laboratories; CoA and ATP, Pabst Laboratories; ergothioneine, L. Light and Company; pyridoxal phosphate, California Foundation for Biochemical Research; Versene, Bersworth Chemical Company; Tiron (disodium-1,2-dihydroxybenzene-3,5-disulfonate), LaMotte Chemical Products Company.

Antimycin A and DPNH-cytochrome *c* reductase flavin (3) were gifts from Dr. Henry R. Mahler, quinacrine from Dr. T. West, P^{32}-labeled pyrophosphate (6) from Dr. Sterling Chaykin, lipoic acid from Eli Lilly and Company, lipothiamide (7) from Dr. Lester Reed, riboflavinyl glucoside (8) from Dr. L. G. Whitby.

Riboflavin-4,5-phosphate was synthesized by the method of Forrest and Todd (9). FAD and FAD-X were prepared according to Dimant *et al.* (10, 11). Toxoflavin was partially purified by the method of van Veen and Mertens (12). We are indebted greatly to Professor van Veen for supplying us with cultures of *Pseudomonas cocovenen* (*Bacterium bongkrek*), which were used as a source of toxoflavin.

Preparations—DPNH oxidase was prepared from pig heart essentially by the method of Sanadi *et al.* (5) for KGD, except that in the final step 35 gm. of ammonium acetate were added per 100 ml. of solution, the precipitate discarded, and 20 gm. additional added to precipitate the enzyme.[2] Specific activities of KGD for the various preparations of enzyme ranged between 60 and 80 (5).[3] In one preparation, the ratio of units of KGD activity to units of DPNH oxidase activity was determined at all stages of the preparation, including a final step of three ammonium acetate fractions:

[2] Dr. Sanadi has informed us that the specific activity of KGD does not vary greatly when precipitated over a wide range of ammonium acetate concentration in the final step of their preparation.

[3] 1 unit of enzyme activity is defined as that amount which liberates 1 μmole of CO_2 per hour in the standard ferricyanide assay system or reduces 0.107 μmole of indophenol per hour in the standard indophenol assay (5).

0 to 31, 32 to 42, and 43 to 54 gm. per 100 ml. of solution. The ratio did not vary greatly, since the extreme values were 0.9 and 1.5.

Crude concentrates of the natural cofactor were prepared as follows: 500 gm. of fresh tissue (heart, kidney, liver, bakers' yeast, or erythrocytes) were boiled in 4 volumes of water for 10 minutes, and the supernatant fluid was recovered after removal of the denatured protein by centrifugation. 1 part (weight by volume) of ammonium sulfate was added, and the solution was extracted three times with 0.2 volume of *n*-butanol. The pale, yellow-brown butanol extracts were pooled, clarified by centrifugation, mixed with 1 volume of chloroform, and extracted three times each with 0.2 volume of 2 N NH_4OH. The aqueous fractions were pooled, extracted three times each with 1 volume of chloroform, which was discarded, and the aqueous fraction was evaporated under reduced pressure (temperature less than 40°) until traces of organic solvents had been removed. These concentrates of the cofactor were contaminated with NH_4^+ which has stimulatory activity of its own, and hence a control isolation, with omission of the tissue, was carried out simultaneously.

Methods—DPN and DPNH were assayed with alcohol dehydrogenase. DPNH modification product (13) was determined by differential spectrophotometry (340 and 290 mμ). Protein concentration was estimated by the biuret technique (14), with bovine serum albumin as the standard and checked by dry weight determinations on dialyzed samples of the enzyme. Fe^{++} (15) and Cu^{++} (16) were determined by published methods.

Assays of KGD were carried out by the ferricyanide or indophenol method (5) with the same units of enzyme activity and specific activity.

The standard spectrophotometric assay system for DPNH oxidase consisted of the following components: 0.2 μmole of DPNH, 500 μmoles of phosphate buffer, pH 7.5, and 0.025 μmole of methylene blue in a volume of 3.0 ml. This mixture was placed in a cuvette of 1 cm. light path and read against a blank (DPNH omitted) at 340 mμ in a Beckman spectrophotometer, model DU. The reaction was started by adding 0.02 ml. of enzyme (about 0.25 mg. of protein) and following the decrease of log I_0/I with time. Δ_{10} is the total change in the log I_0/I for the first 10 minute interval after adding enzyme. 1 unit of DPNH oxidase is defined as that amount causing a change in log I_0/I of 0.010 during the 1st minute.

Results

Spectrophotometric Demonstration of DPNH Oxidation—It was observed initially that DPNH disappeared slowly from the reaction mixtures containing only buffer and the enzyme. The rate of this disappearance could be enhanced greatly by the addition of methylene blue. A typical experiment is illustrated in Fig. 1, where the concentration of DPNH, as shown

by the light absorption at 340 mμ, is plotted as a function of time. In the presence of methylene blue and buffer alone, DPNH was stable. At point *A*, the addition of enzyme to the reaction mixture caused an immediate decrease in the DPNH. At point *B*, the reaction was stopped by heat deproteinization, the pH adjusted to 9.5, and, upon the addition of ethanol and crystalline alcohol dehydrogenase, the light absorption returned immediately to its initial value. This demonstrated that the disappearance of DPNH, in the primary reaction, was not due to its destruction or conversion to DPNH modification product (13, 17). If enzyme and DPNH

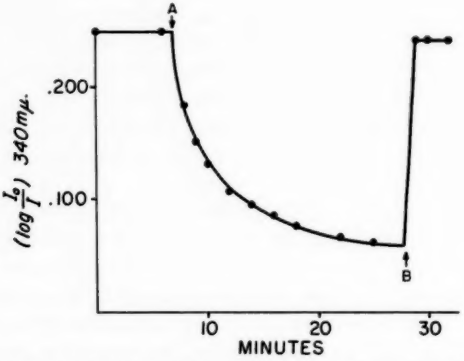


FIG. 1. Oxidation of DPNH by KGD. The standard DPNH oxidase assay system (see "Experimental") was employed, although with less DPNH (0.12 μmole) than usual. At point *A*, 0.02 ml. of enzyme (0.25 mg. of protein) was added. At point *B*, 300 μmoles of Tris buffer, pH 9.5, were added, the solutions transferred to 15 ml. centrifuge cones, boiled for 5 minutes, centrifuged, and the supernatant fluid returned to the cuvettes. 250 μmoles of ethanol and 0.01 ml. (0.6 mg. of protein) of crystalline alcohol dehydrogenase were added at this point to the solutions.

are incubated together in the absence of methylene blue, there is a slow, but perceptible, initial rate with most preparations, indicating the presence of trace amounts of some cofactor, or carrier, in the preparation. In all cases the rate is accelerated greatly by the addition of methylene blue.

Manometric Demonstration of DPNH Oxidation—The above reaction could be carried out manometrically with larger amounts of DPNH. In each of the experiments reported in Table I, 5.4 μmoles of DPNH were subjected to oxidation by molecular oxygen in the presence of enzyme and cofactor. At the end of this time, all of the DPNH had disappeared, but could not be accounted for by the appearance of an equivalent amount of DPN. However, when the solutions were analyzed by differential spectrophotometry for DPNH modification product, it was found that the amount of this

substance which had been formed accounted satisfactorily for the difference.[4]

In spectrophotometric experiments, the addition of catalase does not change the rate of reaction. On the other hand, catalase is essential for the maintenance of the manometric system, owing to the prolonged exposure of enzyme to larger amounts of H_2O_2. H_2O_2 production is shown by the fact that when ethanol was added to the system (Experiment 2) the oxygen uptake was doubled, which is consistent with the stoichiometry imposed by Equations 1 and 2.

TABLE I

Manometric Balance Study of DPNH Oxidation

The complete system in Experiment 1 contained 0.3 ml. of enzyme (3.6 mg. of protein) and 0.3 ml. of 5 per cent bovine serum albumin in the side arm; 5.4 μmoles of DPNH, 0.1 ml. of catalase, 100 μmoles of phosphate buffer, pH 7.0, 0.5 ml. of 10 M ammonium acetate, and water to make 3.0 ml. in the main compartment; 0.2 ml. of 5 M KOH in the center well; O_2 in the gas phase; temperature 37°. The cups were gassed and equilibrated for 20 minutes before the contents were tipped in from the side arm. Duration of experiment, 80 minutes. In Experiment 2, 100 μmoles of ethanol were added to the main compartment. In both experiments, the O_2 uptake was corrected for the appropriate blank less DPNH.

Experiment No.	DPNH	O_2	DPN	DPNH modification product	DPN + DPNH modification product
	μmoles	*μmoles*	*μmoles*	*μmoles*	*μmoles*
1	−5.4	1.9	2.9	2.0	4.9
2	−5.4	3.9	3.2	2.1	5.3

$$H^+ + DPNH + \tfrac{1}{2} O_2 \rightarrow DPN^+ + H_2O \quad (1)$$

$$H^+ + DPNH + O_2 + CH_3CH_2OH \rightarrow DPN^+ + CH_3CHO + H_2O \quad (2)$$

The lack of agreement between oxygen and DPN values in each experiment is unexplained at present, but may be referable to a difficulty in assaying for DPN in the presence of DPNH modification product.

Thus, the balance study of Table I shows that oxygen can serve as the terminal electron acceptor for the oxidation of DPNH to DPN and that hydrogen peroxide is the product. Because of the greater sensitivity and

[4] In unpublished experiments, carried out in collaboration with Dr. E. G. Krebs and Dr. Sterling Chaykin, we have observed that the formation of DPNH modification product occurs under conditions of this experiment; namely, exposure of DPNH to relatively large amounts of the enzyme for long periods of time. In the short term, spectrophotometric experiments, however, essentially no DPNH modification product is formed.

adaptability to routine use, the spectrophotometric assay has been employed throughout the course of this investigation.

Electron Acceptors—In addition to the standard test system with oxygen as the terminal electron acceptor, the oxidation of DPNH can be linked with the net reduction of dyes such as methylene blue and 2,6-dichlorophenolindophenol in anaerobic Thunberg systems. Likewise, indophenol may be used aerobically and the reaction followed in a spectrophotometer at 600 mμ. We have been unable to link this system with the reduction of cytochrome *c*, even in the presence of added flavins and various metal ions such as Cu^{++}, Fe^{+++}, and also MoO_3, which have been shown to be effective in the case of certain flavoproteins (18). Thus, the system appears to be a true diaphorase, rather than a cytochrome *c* reductase with diaphorase activity.

Kinetic Aspects of System—The effect of pH, substrate and cofactor concentration, and enzyme concentration have been investigated briefly. These results are of interest not only as they pertain to the characterization of the DPNH oxidase, but also since they serve to define optimal conditions of the enzyme which may be of use in the assay of any cofactor or carrier for this reaction.

The pH optimum falls between 10.0 and 10.5, as shown in Fig. 2, *A*. There is little difference in activity when the reaction is run in phosphate or glycine buffers, but Tris and Veronal buffers are moderately inhibitory. For most assay purposes, we have chosen to run the reaction at pH 7.5 in order to take advantage of a slower rate, obtainable without excessive dilution of the enzyme.

The activity of the system has been measured as a function of the concentration of DPNH within the rather narrow limits imposed by the spectrophotometric assay (see Fig. 2, *B*). K_m for DPNH has been determined from this curve by the conventional Lineweaver-Burk method (19) and is 4.4×10^{-5} M at 30°. DPNH is completely inactive in this system, while DPN is inhibitory at high concentrations.

With methylene blue as "cofactor," the effect of its concentration upon rate is shown in Fig. 2, *C*; the K_m value in this case is 2.3×10^{-5} M. Finally, the effect of enzyme concentration on reaction rate is illustrated in Fig. 2, *D*.

When the data of a typical run (see Fig. 1) are plotted according to first order kinetics, a straight line is obtained over the first half of the reaction, the rate thereafter declining.

Effect of Inhibitors—Various inhibitors, many of which have been shown in other investigations to inhibit diaphorases and cytochrome *c* reductases, have been tested in this system. Representative results for the more potent inhibitors are presented in Table II, where the per cent inhibition is listed at several different concentrations of inhibitor. Of the anions, ar-

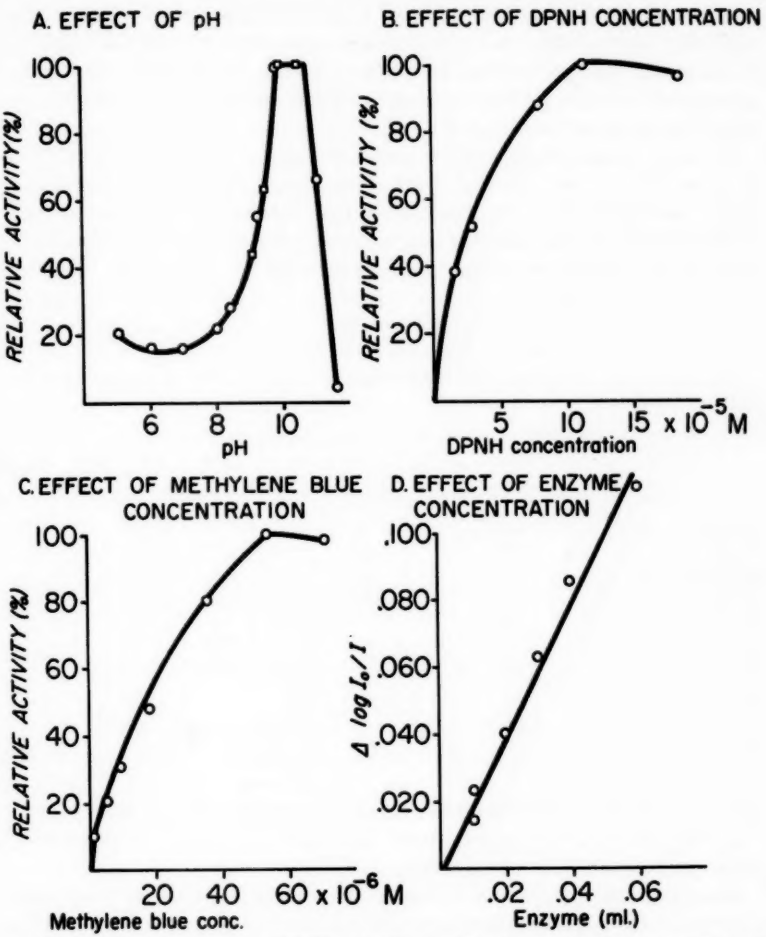


FIG. 2. Kinetic aspects of DPNH oxidation. All determinations were carried out by the standard DPNH oxidase assay, and the results are expressed as relative activity in per cent (optimal activity taken as 100). In *D*, however, the ordinate is expressed in absolute units of activity; namely, the change in log I_0/I for the 1st minute. *A*, standard assay conditions, with the pH as indicated. 200 μmoles of the following buffers were present: ○ = phosphate; □ = glycine. The pH was measured with a glass electrode at the conclusion of each experiment. *B* and *C*, standard assay conditions; final concentrations of DPNH and methylene blue as indicated. *D*, standard assay conditions (except that the initial amount of DPNH was increased to 0.4 μmole); enzyme concentration as indicated. 0.01 ml. of enzyme contained 0.12 mg. of protein.

senate and cyanide are inhibitory at high concentrations (10^{-2} M). The inhibition by PCMB at very low concentrations (10^{-5} to 10^{-6} M) may be reversed by cysteine. Chloride, nitrate, sulfate, iodoacetate, and pyrophosphate show no inhibition, while arsenite provides some stimulation, as yet unexplained.

Divalent cations, such as Ba^{++} and Zn^{++}, and, to a lesser extent, Ca^{++} (but not Mg^{++}, Mn^{++}, or Cu^{++}) are moderately inhibitory. The relative lack of inhibition by Cu^{++} when the enzyme is assayed with either oxygen or indophenol as the terminal electron acceptor is especially significant since other diaphorases (2) are inhibited strongly by this metal. Hg^{++} is

TABLE II

Effect of Inhibitors on DPNH Oxidase

Standard assay conditions for DPNH oxidase, with inhibitor at the final concentration indicated.

Inhibitor	Per cent inhibition			
	10^{-2} M	10^{-4} M	2×10^{-5} M	5×10^{-6} M
Arsenate	80	5		
Cyanide	75	19		
Iodosobenzoate		22		
PCMB		100	40	
Hydroxylamine	26			
Hg^{++}		100	100	12
Ba^{++}	63	39		
Zn^{++}	39			
Na^{+}	51	4		
Ag^{+}		63		

one of the most powerful inhibitors studied. Ag^{+} is very inhibitory, Na^{+} is moderately so, K^{+} is not inhibitory, and NH_4^{+} provides a curious stimulation, to be discussed subsequently.

Other substances which were without effect include quinacrine (a general inhibitor of dissociated flavoproteins (20)), 2,4-dinitrophenol and gramicidin (inhibitors of oxidative phosphorylation (21)), BAL, and antimycin A (an inhibitor of certain electron transport systems (22)). Hydroxylamine was moderately inhibitory at high concentration.

Relation to Oxidative Phosphorylation—It should be noted briefly that the standard assay system described above does not carry out oxidative phosphorylation during the oxidation of DPNH. The experiments were carried out by adding to the usual components either (*a*) P^{32}-labeled orthophosphate plus ADP or (*b*) P^{32}-labeled pyrophosphate plus AMP. In both cases, after oxidation of DPNH had occurred, the reaction mixture was

deproteinized by heating, concentrated by lyophilization, and examined for P^{32}-labeled ATP by ion exchange chromatography on Dowex 1 according to the method of Cohn and Carter (23), or paper chromatography in acetone-water (2:1), propanol-concentrated NH_3-water (6:3:1), ethanol-0.1 M sodium acetate, pH 7 (7:3), or isobutyric acid-concentrated NH_3-water (66:1:33). Under these conditions there appeared to be no incorporation of radioactivity into any organic phosphate fraction.

Relation of DPNH Oxidase to KGD—It is of interest to compare the DPNH oxidase to the KGD activity found in the same preparation. From the data of Sanadi *et al.* (5, 24) it may be calculated that 20 units of enzyme catalyze the oxidation of KG at the rates shown in Table III when indophenol, ferricyanide, and DPN, respectively, are used as the electron ac-

TABLE III

Multiple Enzymatic Activities Associated with KGD

All assays carried out as described in "Experimental" except that the DPNH oxidase assay was run at pH 10.

Assay method			Velocity, μmoles per ml. per min. × 10^2	
			Sanadi *et al.*	Present investigation
α-Ketoglutaric dehydrogenase.		Indophenol.	4.5	4.2
"	"	Ferricyanide	1.8	2.2
"	"	DPN.....	14.4	
DPNH oxidase.....				16.3

ceptors. Since each of these three acceptors involves a different assay system, the *maximal* velocities in each case are compared.

Using a comparable amount of KGD, we have obtained results for the indophenol and ferricyanide systems (second column of Table III) in good agreement with the previous data (5, 24). The rate of oxidation of DPNH carried out by this preparation,[5] is equal roughly to the maximal rate of reduction of DPN, indicating that the diaphorase activity is certainly significant and that it could well be a part of an over-all electron transport system, since KGD carries out the reaction

$$KG + CoA + DPN^+ \rightarrow \text{succinyl CoA} + CO_2 + DPNH + H^+ \quad (3)$$

Except for the final step in the preparation, in which we have found that KGD of the highest specific activity precipitates at a slightly lower am-

[5] Preparations of KGD in Dr. Sanadi's laboratory show a considerably lower level of DPNH oxidase activity (5) than that reported here, especially after additional purification steps such as precipitation at pH 5.9 or ultracentrifugation (private communication).

monium acetate concentration than was reported by Sanadi *et al.* (5),[2] the specific activities and total recoveries at each step are in good agreement with the findings of these authors. At each step, the KGD activity, determined by indophenol assay, and the DPNH oxidase activity have been checked. The activities are concentrated in a parallel manner during purification, including the final step in which the enzyme may be fractionated by serial precipitation with ammonium acetate (see "Experimental").

Sanadi *et al.* (5) have reported that their most purified preparations (specific activity = 80) of KGD are essentially homogeneous when examined electrophoretically, but display a small second peak in the ultracentrifuge. We have confirmed, in part, these observations with our preparations[6] and have found in the electrophoresis apparatus, for example, a single peak (about 90 per cent of the total protein) with a mobility of -5.8×10^{-5} sq. cm. per volt second in 0.05 M phosphate buffer, pH 7.5 (5). However, in the ultracentrifuge, our preparations have two components in the same phosphate buffer. This is similar to the findings of the above authors, but differs in that the two components are approximately equal in amount. After selective heat treatment (5 minutes at 50°, see below) and removal of the denatured material, the heavier component and the KGD activity are absent, while the lighter component and the DPNH oxidase activity are still present. Likewise, ultracentrifugation (30,000 × *g* for 1 hour) of KGD preparations brings down a gelatinous pellet, which is difficult to redissolve but which contains the KGD activity and leaves a supernatant fluid containing considerable amounts of DPNH oxidase activity and no KGD activity. Further studies of the physical properties of KGD and of the partially heat-denatured preparation would be necessary to decide whether the DPNH oxidase activity is an integral part of the KGD (*i.e.*, a separate active center on the macromolecule) or a smaller molecular weight protein loosely associated with the macromolecule. If the latter case should prove to be true, it is difficult to understand how our preparations of KGD can contain appreciable amounts of a separate DPNH oxidase, yet possess such a high *specific activity* of KGD.

The effect of partial heat denaturation upon the two enzymatic activities is illustrated in Fig. 3. Aliquots of the preparation were heated at the indicated temperature for 5 minutes, cooled, the enzymatic activity measured immediately, and compared to the control which had been kept at 0°. KGD, tested in the indophenol assay system, is relatively heat-labile, since all activity is lost by heating at 60°. On the other hand, the DPNH oxidase activity is quite heat-stable, since activity is retained until the enzyme is heated above 90°. An unexplained but reproducible rise in the latter activity occurs when the preparation is heated between 75° and 85°.

[6] We are indebted to Dr. H. Neurath for making these facilities available and to Mr. Roger Wade for carrying out the determinations.

Additional evidence bearing upon differences between the two catalytic activities was obtained from the effect of PCMB upon the preparation. The DPNH oxidase is depressed to the extent of 50 per cent at about 1.6×10^{-5} M and completely inhibited at 10^{-4} M; the inhibition is reversed by the addition of 10 μmoles of cysteine. On the other hand, KGD is strongly inhibited by very low concentrations of PCMB; *i.e.*, 50 per cent inhibition at 5×10^{-6} M. It has been shown previously that this latter inhibition is not reversed by cysteine (5).

Requirement for Cofactor—With the spectrophotometric assay for DPNH oxidation described previously, a variety of known cofactors have been screened for the ability to replace methylene blue as a cofactor or carrier in

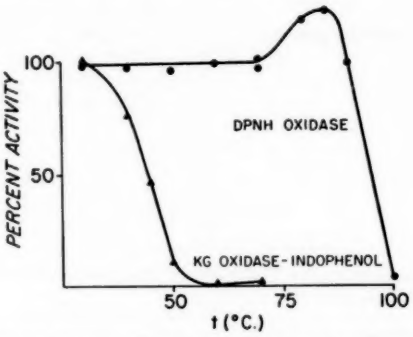


FIG. 3. Effect of heat denaturation. Aliquots of the enzyme were exposed to the indicated temperature for 5 minutes, immediately chilled, and the denatured protein removed by centrifugation. Assays for KGD were carried out by the indophenol method, and for the DPNH oxidase by the standard method.

this system. Special attention has been directed toward the flavin nucleotides, since these coenzymes often mediate the electron transfer between reduced pyridine nucleotides and cytochrome *c* or oxygen. Riboflavin, riboflavinyl glucoside (8), FMN, 4,5-FMN (cyc.) (9), FAD, FAD-X (11), and the DPNH-cytochrome *c* reductase flavin (3) were all either completely inactive or slightly inhibitory. Other substances, which likewise proved to be ineffective, were oxidized and reduced CoA, cysteine, cystine, oxidized and reduced glutathione, ergothioneine, biotin, pyridoxal phosphate, diphosphothiamine, folic acid, lipoic acid, and lipothiamide (7).

Certain oxidation-reduction substances, however, were able to serve as the cofactor. As shown in Table IV, Cu^{++} produced a measurable stimulation, which was enhanced in the presence of FAD. Cu^{+}, ascorbate, and molybdate showed slight effects, while Co^{++}, Fe^{+++}, and Fe^{++} were inactive. Toxoflavin and 2-methyl-1,4-naphthoquinone were effective at low concentrations, although the exact concentration dependence, and hence the

optimal level, was not ascertained as in the case of methylene blue (see Fig. 2, *C*).

TABLE IV

Activity of Various Cofactors

Experimental conditions as in Fig. 1 except that methylene blue was replaced by the indicated cofactor. All results have been corrected for the non-enzymatic oxidation of DPNH by the cofactor, which was small in all cases except for Fe^{++}.

Cofactor	Concentration	Δ_{10}
	M	
Methylene blue	5.4×10^{-6}	0.322
Toxoflavin	*Ca.* 10^{-5}	0.195
2-Methyl-1,4-naphthoquinone	3.3×10^{-5}	0.134
FAD	1.7×10^{-6}	0.000
Cu^{++}	3.3×10^{-4}	0.103
FAD + Cu^{++}	1.7×10^{-6} + 3.3×10^{-4}	0.123

TABLE V

Effect of ATP Concentration

Experimental conditions as in Fig. 1 except that methylene blue was replaced by the *amount* of ATP indicated.

ATP added	Δ_{10}
μmoles	
1	0.015
5	0.013
10	0.022
25	0.046
25*	0.041
25†	0.030
50	0.066
100	0.071

* Pabst ATP.

† Sigma ATP, crystalline; all other values obtained with commercial Sigma ATP.

Effect of Adenine Nucleotides—In connection with the data accompanying Fig. 1, it was stated that the enzyme without added methylene blue has slight oxidase activity, indicating the presence, presumably, of small amounts of some natural cofactor. In the absence of any other added cofactor, this endogenous effect can be considerably enhanced by the addition of ATP. In Table V, the stimulation produced by increasing amounts of ATP is recorded. It should be noted that rather large amounts of ATP

are required, and that the effect levels off at a Δ_{10} of about 0.070. It is possible that the ATP is contaminated with trace amounts of some other actual cofactor, since similar results are obtained with two different commercial samples of ATP, whereas lower activity is obtained with crystalline ATP.

Among compounds of a similar nature which were tested for this stimulation, only ITP was found to be effective. The stimulation afforded by this substance exceeded slightly that of a comparable amount of ATP. ADP had a very small effect, while adenylic acids *a*, *b*, and 5′, acetyl phosphate, pyrophosphate, adenine, and adenosine were completely devoid of activity.

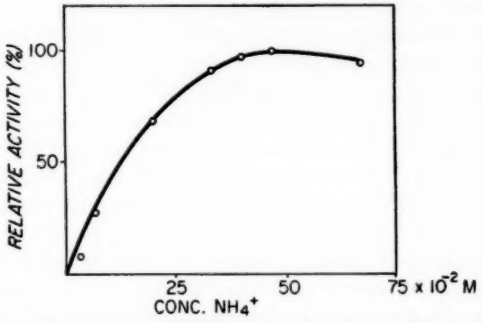


FIG. 4. Effect of NH_4^+ concentration. Experimental conditions as in Fig. 1 except that methylene blue was omitted. Activity was measured as Δ_{10} and then converted to relative activity, the optimal activity being considered as 100 per cent. NH_4^+, added as NH_4Ac, is expressed as the final concentration in the cuvette.

Effect of NH_4^+—While investigating the effect of various ions as inhibitors, we observed that NH_4^+ actually gave a small stimulation at the level tested (about 2×10^{-2} M). When the concentration was raised, larger effects were recorded (Fig. 4) until at the optimal level (about 50×10^{-2} M) the stimulation compared favorably with that afforded by methylene blue (see Fig. 2, *C*).

The system was relatively insensitive to the anion, since ammonium acetate, sulfate, nitrate, and chloride were all active. Sodium and potassium salts at similar concentrations were completely inactive.

It was thought that the large amounts of NH_4^+ required might be indicative that a trace impurity, perhaps a metal, was actually being supplied as the cofactor. However, the activity was not depressed in the presence of Versene, Tiron, or diethyl dithiocarbamate, nor could any characteristic color be developed indicative of Fe^{+++} and Cu^{++}, respectively, when the latter two reagents were added to high concentrations of the ammonium

salts. Hydrazine and hydroxylamine, which are possible contaminants of ammonium compounds, were tested individually and found to be devoid of activity. The ammonium salts could be recrystallized from hot water and ethanol without any decrease in activity. A similar stimulation of horse liver aldehyde oxidase by NH_4^+ has been reported by Carpenter (25).

Evidence for Naturally Occurring Cofactor—The existence of a small amount of endogenous cofactor in the enzyme preparation led to the search in various tissues for a naturally occurring cofactor. By means of the spectrophotometric assay system, it was found that extracts of liver, heart, kidney, bakers' yeast, and erythrocytes do contain a stimulatory substance. Preliminary studies directed toward the purification of this material has shown that it is both heat-stable and dialyzable. From aqueous solutions, saturated with ammonium sulfate, it can be extracted into *n*-butanol and back-extracted into alkaline aqueous solution by the addition of chloroform. Ethyl acetate and *n*-amyl alcohol can be substituted for butanol in these extractions. The solubility of the factor in both aqueous and organic solvents is consistent with its behavior on paper chromatograms. When a concentrate of the factor was chromatographed with phosphate buffer of pH 8 (5 per cent $Na_2HPO_4 \cdot 7H_2O$), most of the activity appeared between R_F 0.60 and 0.70, and a smaller amount between R_F 0.50 and 0.60. In *n*-butanol saturated with water, the activity again migrated well up the paper, with a peak between R_F 0.70 and 0.80.

Concentrates of the cofactor are pale yellow in color, with a yellow fluorescence of much less intensity than that of the flavins. The absorption spectra of these solutions is characterized by a peak at 400 mμ. It is of interest that toxoflavin, a naturally occurring pigment from the microorganism *P. cocovenen*, serves also as a cofactor in this system (see Table IV), is likewise characterized by an absorption maximum at 400 mμ, and was purified by van Veen and Mertens (12) using similar procedures of extraction into butanol, ethyl acetate, and amyl alcohol. On the other hand, toxoflavin forms a red addition product with bisulfite, but we have not observed this effect with our material. Recently, Edson and Cousins (26) have purified a lactic oxidase from *Mycobacterium smegmatis*, which has as a prosthetic group, a substance whose properties and absorption spectrum are similar to the above compounds.

DISCUSSION

It is of interest to compare the properties of this enzyme system, including its cofactor requirements, with those of other diaphorases isolated from the same tissue.

The classical diaphorase was isolated first as a particulate preparation by Dewan and Green (27) and by von Euler and Hellström (28), and later made soluble and extensively purified by Straub (2). This enzyme, which

has FAD as its prosthetic group, bears some resemblance to the enzyme described in this paper, especially with regard to the tissue of origin, heat stability, requirement for methylene blue as a cofactor, and stimulation by NH_4^+ (unpublished observation in this Laboratory). The properties of Straub's diaphorase have never been documented extensively, but, since it has a lower pH optimum of 8.5, a yellow fluorescence, a sensitivity to various inhibitors, and contains a flavin but no other component, it would not appear to be identical with the enzyme described in this paper. It appears that the present enzyme does not contain any flavin on the basis of its lack of fluorescence or light absorption at 450 mμ, but further study is necessary to verify this point.

Although DPNH-cytochrome *c* reductase (29) will act as a diaphorase, it has a flavin prosthetic group (3), and, moreover, the sensitivity to various inhibitors, notably Cu^{++} (4), differentiates it from the present system.

In many respects, the DPNH oxidase described here resembles the methemoglobin reductase, which we have purified extensively from human erythrocytes (30). This enzyme oxidizes TPNH (and to a lesser extent DPNH) by oxygen or methemoglobin, requires methylene blue as a carrier, does not appear to contain flavin, but has an absorption band at 410 mμ. Its sensitivity to inhibitors closely resembles that of the present enzyme.

It was noted by Sanadi *et al.* (5) that purified preparations of KGD, even after prolonged dialysis, are distinctly yellow or orange and possess an absorption maximum at 410 mμ. These characteristics may be due, of course, to traces of flavin and iron porphyrin impurities. In our enzyme preparations the ratio of light absorption at 280:410 mμ is surprisingly constant, ranging from 11.7 to 12.3. Since the peak at 410 mμ persists even after all KGD activity has been removed by selective heat denaturation at 50–60°, it is possible that the present DPNH oxidase contains an iron porphyrin prosthetic group.

The authors are indebted to Dr. D. R. Sanadi for his comments and suggestions pertaining to this problem.

SUMMARY

1. A DPNH oxidase, or diaphorase, which has been purified from pig heart, catalyzes the oxidation of DPNH, but not TPNH, at the expense of O_2. The reaction may be followed spectrophotometrically by the reduction of methylene blue or indophenol or manometrically by the uptake of O_2.

2. Optimal conditions of enzyme, substrate and cofactor concentration, and pH, have been established, and the appropriate kinetic constants were determined.

3. The enzyme is inhibited by Hg^{++}, Zn^{++}, Ba^{++}, Ag^{+}, Na^{+}, arsenate, CN^{-}, and PCMB.

4. During the aerobic oxidation of DPNH, the enzyme does not carry out oxidative phosphorylation.

5. DPNH oxidase has been found to be associated with α-ketoglutaric dehydrogenase during purification. Although the two enzymes cannot be distinguished in the electrophoresis apparatus at pH 7.5, they differ in their behavior in the ultracentrifuge, in their sensitivity to *p*-chloromercuribenzoate, and in their heat stability.

6. Various substances such as dyes, toxoflavin, quinones, and Cu^{++} have been shown to serve as cofactors for the DPNH oxidase. The existence of a naturally occurring, unidentified cofactor has been demonstrated. In an unexplained manner, both ATP and NH_4^{+} can partially replace methylene blue as cofactor.

7. The relationship of this enzyme to other diaphorases has been discussed, with the conclusion that it most resembles the methemoglobin reductase of erythrocytes.

BIBLIOGRAPHY

1. Huennekens, F. M., and Basford, R. E., *Federation Proc.*, **13,** 232 (1954).
2. Straub, F., *Biochem. J.*, **33,** 787 (1939).
3. Mahler, H. R., Sarkar, N. K., Vernon, L. P., and Alberty, R. A., *J. Biol. Chem.*, **199,** 585 (1952).
4. Vernon, L. P., Mahler, H. R., and Sarkar, N. K., *J. Biol. Chem.*, **199,** 599 (1952).
5. Sanadi, D. R., Littlefield, J. W., and Bock, R. M., *J. Biol. Chem.*, **197,** 851 (1952).
6. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **191,** 535 (1951).
7. Reed, L., *Physiol. Rev.*, **33,** 544 (1953).
8. Whitby, L. G., *Biochem. J.*, **50,** 433 (1951-52).
9. Forrest, H. S., and Todd, A. R., *J. Chem. Soc.*, 3295 (1950).
10. Dimant, E., Sanadi, D. R., and Huennekens, F. M., *J. Am. Chem. Soc.*, **74,** 5440 (1952).
11. Huennekens, F. M., Sanadi, D. R., Dimant, E., and Schepartz, A. I., *J. Am. Chem. Soc.*, **75,** 3611 (1953).
12. van Veen, A., and Mertens, W., cited in Oppenheimer, C., and Stern, K., Biological oxidations, New York, 233-237 (1939).
13. Rafter, G. W., Chaykin, S., and Krebs, E. G., *J. Biol. Chem.*, **208,** 799 (1954).
14. Weichselbaum, T., *Am. J. Clin. Path.*, **7,** 40 (1946).
15. Yoe, J., and Jones, A., *Ind. and Eng. Chem., Anal. Ed.*, **16,** 111 (1944).
16. Chow, T., and Thompson, T., Technical Report No. 9, Office of Naval Research, contract N8onr-520/III, project NR-083-012.
17. Krebs, E., Abstracts, American Chemical Society, 123rd meeting, Los Angeles, 18C, Mar. (1953).
18. Mackler, B., Mahler, H. R., and Green, D. E., *J. Biol. Chem.*, **210,** 149 (1954). Mahler, H. R., and Elowe, D. G., *J. Biol. Chem.*, **210,** 165 (1954). Mahler, H. R., Mackler, B., Green, D. E., and Bock, R. M., *J. Biol. Chem.*, **210,** 465 (1954).
19. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
20. Hellerman, L., Lindsay, A., and Bovarnick, M. R., *J. Biol. Chem.*, **163,** 553 (1946).

21. Cross, R. J., Taggart, J. V., Covo, G. A., and Green, D. E., *J. Biol. Chem.*, **177**, 655 (1949).
22. Potter, V. R., and Reif, A. E., *J. Biol. Chem.*, **194**, 287 (1952).
23. Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72**, 4273 (1950).
24. Sanadi, D. R., and Littlefield, J. W., *J. Biol. Chem.*, **201**, 103 (1953).
25. Carpenter, F., *Acta chem. Scand.*, **5**, 406 (1951).
26. Edson, N., and Cousins, F., *Nature*, **171**, 702 (1953).
27. Dewan, J., and Green, D., *Biochem. J.*, **31**, 1069 (1937); **32**, 626 (1938).
28. von Euler, H., and Hellström, H., *Z. physiol. Chem.*, **252**, 31 (1938).
29. Mahler, H. R., and Elowe, D. G., *J. Am. Chem. Soc.*, **75**, 5769 (1953).
30. Huennekens, F. M., and Gabrio, B. W., *Federation Proc.*, **13**, 232 (1954).

STUDIES IN STEROID METABOLISM

III. METABOLISM OF TESTOSTERONE BY HUMAN SERUM*

By HERBERT H. WOTIZ, ROLAND RICHTERICH-VAN BAERLE, AND HENRY M. LEMON

With the Technical Assistance of Rose Fishman

(From the Departments of Biochemistry and Medicine, Boston University School of Medicine, Boston, Massachusetts)

(Received for publication, June 15, 1954)

Among the more puzzling aspects of endocrine research today is the inability of investigators to detect any testosterone in peripheral blood. The only exception to this is the isolation by West *et al.* (1) of testosterone from spermatic vein blood of the dog. These findings are of particular interest, inasmuch as some of the more labile corticosteroids have been found in human peripheral blood (2).

This inability to detect testosterone in peripheral blood might be explained by the rapid metabolism of this steroid by blood as well as by other tissues. These catabolic reactions were assumed to be located primarily in the liver. Samuels (3, 4) and his coworkers were able not only to demonstrate the metabolism of testosterone by liver and kidney of human and animal origin, but also to characterize one of the enzymes involved (5). None of the other tissues tested showed any evidence of ability to metabolize testosterone. West (6, 7) and associates, using hepatectomized and nephrectomized animals and then humans with liver disease, demonstrated indirectly that testosterone must also be metabolized by tissues other than the liver and kidney. The metabolic effect on testosterone by human prostate (8, 9) and several other tissues (10), particularly skin and breast cancer, was recently demonstrated in this laboratory.

During the last decade several enzymes have been found in human serum; *e.g.*, phosphatases, glucuronidase, and cholinesterase. An investigation was carried out as to the possibility of the presence in human serum of the widely distributed enzyme system concerned with the metabolic conversion of testosterone. In a preliminary report the presence of such an enzyme system in human serum has already been described (11). The characteristics of this enzyme system will be presented here and its physiological significance discussed.

* These studies have been carried out with the assistance of research grants from the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council, from Aids for Cancer Research (Boston, Massachusetts), and from teaching and research grants from the National Cancer Institute, National Institutes of Health.

EXPERIMENTAL

Incubations—All experiments were carried out on serum prepared from freshly drawn blood from one of the investigators. The serum was utilized within a few minutes after centrifugation. In a typical experiment 1 ml. of serum was added to 20 ml. of Krebs-Ringer-phosphate buffer at pH 7.3 containing 2 mg. of diphosphopyridine nucleotide (DPN) and 2 mg. of testosterone[1] dissolved in 0.1 ml. of ethanol. The mixture was incubated for 3 hours at 38° under aerobic conditions, after which time the flasks and contents were boiled for 10 minutes.

Extraction and Quantitative Measurement—The extraction procedure, as well as quantitative measurement of the residual testosterone, is essentially that reported previously (9), except that the homogenization and acetone extraction were eliminated. Special care must be taken to break up any protein conglomerate, since this frequently occludes steroidal material. This is done best by carefully rubbing the centrifuged proteins with a glass stirring rod while covered with ether. Also, in order to compensate for the increased error in the quantitative measurement due to the decrease in testosterone concentration, 0.1 ml. of the final extract was applied to the paper chromatogram. Thus, the limit of error remained at ±5 per cent.

Hydrolysis—Some of the aqueous residues from these experiments were hydrolyzed by boiling with 20 volumes per cent of hydrochloric acid for 30 minutes; after cooling, they were extracted and analyzed as before.

Results

Extraction of Testosterone from Serum—When freshly drawn serum was shaken once with testosterone and immediately extracted, only 63 per cent of the steroid was recoverable. Dilution of the serum with 10 ml. of buffer prior to the addition of the steroid increased the extractability of the added compound to 74 per cent, and dilution with 20 ml. of buffer increased recovery to 82 per cent. When the extraction procedure as described by West *et al.* (7) was applied (the proteins precipitated with 95 per cent ethanol, centrifuged, and washed four times), the recovery of added steroid was 16 to 22 per cent lower. This suggests a significant loss of testosterone in the discarded protein fraction.

Controls—Several flasks, each containing 1 ml. of serum in 20 ml. of buffer, 2 mg. of DPN, and 2 mg. of testosterone, were boiled for 10 minutes immediately before introduction of the steroid and DPN and extracted after incubation varying from 1 to 16 hours. Recoveries from these experiments varied from 91 to 96 per cent. Further verification of the inhibition of the enzyme system by these procedures was obtained upon

[1] The authors gratefully acknowledge the contribution of testosterone from Organon, Inc.

examination of paper chromatograms. At no time was any steroid other than starting material detectable after staining the paper strips with Zimmermann's reagent.

Bacterial contamination can be excluded for several reasons. The blood was drawn under sterile conditions and used for the incubation within less than 1 hour. The incubation time for most experiments was too short (3 hours) to allow significant growth. Nevertheless, the incubation mixtures were checked after all incubations by means of sediment smears and subsequent staining as well as by cultivation methods. No significant

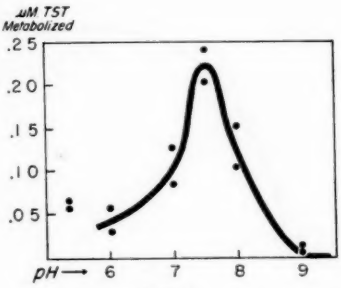


FIG. 1

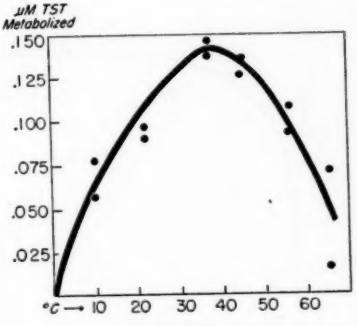


FIG. 2

FIG. 1. Effect of pH on metabolism of testosterone by human serum. Each flask contained 20 ml. of buffer, 2 mg. of testosterone, 2 mg. of DPN, and 1 ml. of serum. Incubation at 38° for 3 hours in air.

FIG. 2. Effect of temperature on metabolism of testosterone by human serum. Each flask contained 20 ml. of Krebs-Ringer-phosphate buffer at pH 7.3, 2 mg. of testosterone, 2 mg. of DPN, and 1 ml. of serum. Incubation at 38° for 3 hours in air.

bacterial contamination could be detected with either method, even in those incubations lasting 24 hours.

The following experiments were conducted to determine optimal conditions of pH, enzyme concentration, substrate concentration, temperature, and incubation time. Two or more determinations were made for all points plotted.

Effect of Varying pH Level—In Fig. 1 are recorded the results obtained from two groups of experiments incubated at various pH levels. Optimal activity is obtained between pH 7.0 and 7.5. At pH 9 all of the added substrate was recovered.

As a result of the heavy precipitation of proteins in buffers of low pH, it was impossible to ascertain whether the elevated values of pH 5.4 would indicate a second peak of activity or whether these values represent a

constant experimental error because of the difficulties, previously mentioned, in extracting testosterone from precipitated serum proteins.

Effect of Temperature Variation—When the temperature is increased from 10° to 65°, optimal activity is reached around 40° (Fig. 2). The curve obtained in these experiments is typical for an enzymatic reaction.

Effect of Varying Substrate Concentration—A series of incubations with amounts of testosterone varying from 0.7 to 7 μmoles was carried out, and the results obtained are plotted in Fig. 3. The optimal substrate concentration is reached at about 3.5 μmoles of testosterone.

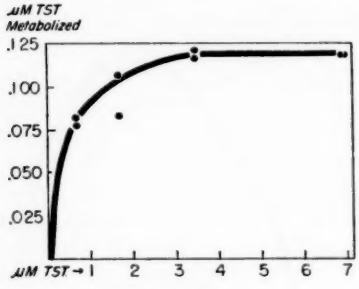


FIG. 3

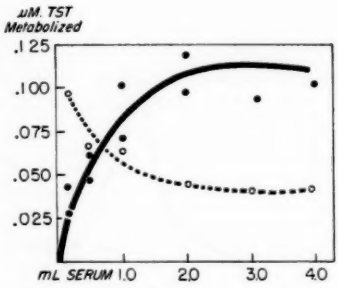


FIG. 4

FIG. 3. Effect of substrate concentration on metabolism of testosterone by human serum. Each flask contained 20 ml. of Krebs-Ringer-phosphate buffer, 2 mg. of DPN, 1 ml. of serum, and varying amounts of testosterone. Incubation at 38° for 3 hours in air.

FIG. 4. Effect of varying serum concentration on metabolism of testosterone by human serum. Dash line, each flask contained 20 ml. of Krebs-Ringer-phosphate buffer, 2 mg. of DPN, 2 mg. of testosterone, and amounts of serum varying from 0.5 to 4.0 ml. Incubation at 38° for 3 hours in air. Solid line, each flask contained 2 mg. of testosterone, 2 mg. of DPN, amounts of serum varying from 0.25 to 4.0 ml. of serum, and buffer to maintain a ratio of 1:20.

Effect of Varying Serum Concentration—Amounts of serum varying from 0.25 to 4.0 ml. were incubated for 3 hours at 38° with 20 ml. of buffer containing 2 mg. of substrate and 2 mg. of DPN. The results of these experiments are plotted in Fig. 4 (dash line). Increased precipitate formation, paralleling the serum concentration, was observed at the end of the incubation period. It was thought that the disturbance of the stability of the suspension might lead to a partial inactivation of the enzyme systems. The experiments were, therefore, repeated, the ratio of buffer to serum (20:1) being kept constant (Fig. 4, solid line). Optimal activity was reached upon incubating 2 ml. of serum in 40 ml. of buffer. The level

attained at this serum concentration remains fairly constant, regardless of any further increase in the amount of serum.

Effect of Varying Time of Incubation—A number of incubations with the standard incubation mixture were carried out for periods varying from 0 to 24 hours. The curve (Fig. 5) is biphasic, and the first maximum occurs at 1 hour. This is followed by a minimum at 2 hours, which gradually increases until a second peak is reached at the end of 16 hours.

In Fig. 6 the paper chromatograms of these incubations are reproduced as they appeared after staining with Zimmermann's reagent. No metabolites

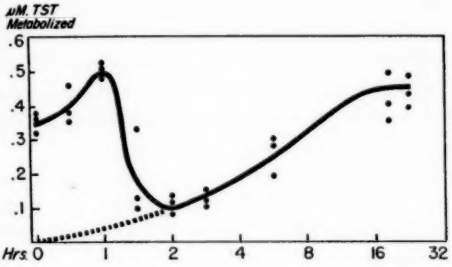


FIG. 5

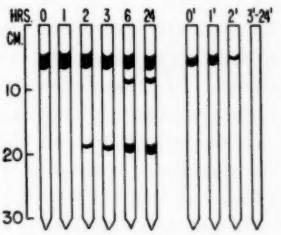


FIG. 6

FIG. 5. Effect of incubation time on metabolism of testosterone by human serum. Each flask contained 20 ml. of Krebs-Ringer-phosphate buffer, 2 mg. of testosterone, 2 mg. of DPN, and 1 ml. of serum. Incubation at 38° for 0 to 24 hours in air.

FIG. 6. Effect of varying time of incubation. Paper chromatograms stained with Zimmermann's reagent after development in ligroin-propylene glycol system. Strips 0 to 24, chromatograms of unhydrolyzed extracts; Strips 0′ to 24′, chromatograms of extracts from aqueous residues after acid hydrolysis.

could be found on incubation of less than 2 hours. From incubations of 2 hours or longer, at least one new substance was detectable. After acid hydrolysis of the aqueous residues from the 0 to 2 hour experiments, a very small amount of testosterone, identified by mixed paper chromatography, could be recovered; no testosterone could be demonstrated with the Zimmermann reaction (lower limit 10 γ of steroid) after hydrolysis of aqueous residues from experiments incubated longer than 2 hours, as shown in Fig. 6 (Strips 0′ to 24′). The new metabolites account for nearly all of the missing testosterone.

The second portion of the curve in Fig. 5 (2 to 24 hours) is typical for an enzymatic reaction; the first peak is probably due to a reversible binding of the testosterone to protein, a linkage strong enough to remain unbroken by acid hydrolysis. This possibly non-enzymatic binding of testosterone

from 0 to 2 hours appears to be superimposed on the enzymatic reaction curve (dash line) for the time interval from 0 to 2 hours.

DISCUSSION

Most of our knowledge of the metabolism of testosterone in humans is derived from urinary studies in normal individuals who have been injected with various steroids. This method has provided us with an over-all picture of the metabolism, but gives no information as to the intermediary products or the site of their conversion. This lack in our knowledge may be overcome by studies with human tissues *in vitro*. Kochakian (12) and Samuels (13) and their coworkers found active metabolism of testosterone by human liver tissues. In cirrhosis of the liver a marked reduction of testosterone conversion was observed. Metabolic degradation of testosterone by human prostate (8, 9), as well as by a number of other human tissues (10), was demonstrated in this laboratory. Androstenedione has been identified as a major metabolite in all instances.

Little is known about the metabolism of human tissues *in vitro* or about the enzymes involved in those conversions. The observation by Richterich-van Baerle *et al.* (11) that human blood serum contains an active testosterone-metabolizing enzyme system offered a convenient way of characterizing a human enzyme system. The optimal pH of this enzyme system is between pH 7.1 and 7.4, within the range of the normal pH of blood serum. The enzymes in question could therefore be active in human serum *in vivo*. Moreover, the optimal temperature is at the physiological level.

Boiling the serum prior to its incubation leads to an inactivation of the enzyme activity. A partial inactivation of the enzyme could also be observed by increasing the serum concentration in a constant amount of buffer. In this type of experiment formation of a protein precipitate was observed after the incubation. The enzymatic activity appears therefore to be bound to the solubility of serum proteins. Attempts to recover the testosterone immediately after its addition to serum, without incubation, resulted in recovery of about 60 per cent of the steroid. Even with acid hydrolysis, only a trace (about 2 per cent) of the missing testosterone could be recovered. The larger the concentration of the serum, the smaller was the amount of testosterone recoverable.

The experiments involving variation of incubation time show remarkable results. Maximal disappearance of testosterone was observed at 1 hour. However, it was never possible to demonstrate the appearance of any metabolites after less than 2 hours incubation. Therefore, the testosterone unaccounted for seems not to have been metabolized. The impossibility of recovering any significant amount of substrate after acid hydrolysis of

the residue can best be explained by a strong but reversible protein binding. This binding is spontaneously reversible, possibly through denaturation of the protein bound to the steroid, because, after standing at 38° for 2 hours, most of the testosterone could be recovered with the exception of the small portion which was metabolized and which could be explained by the appearance of metabolites. Such protein binding was previously observed by others (14–16).

Thus, two different mechanisms must be involved in the interaction of testosterone and human serum. The first represents a very strong binding of the testosterone to a protein, the second, an enzymatic conversion of testosterone to its metabolites.

SUMMARY

1. It has been shown that human serum participates *in vitro* in the metabolism of testosterone.

2. The reaction between serum proteins and testosterone is twofold: first, a reversible binding between the steroid and a protein without formation of metabolites; second, the enzymatic degradation of the steroid molecule.

BIBLIOGRAPHY

1. West, C. D., Hollander, V. P., Kritchevsky, T. H., and Dobriner, K., *J. Clin. Endocrinol. and Metabolism*, **12**, 915 (1952).
2. Nelson, D. H., Samuels, L. T., Willardson, D. G., and Tyler, F. H., *J. Clin. Endocrinol.*, **11**, 1021 (1951).
3. West, C. D., and Samuels, L. T., *J. Biol. Chem.*, **190**, 827 (1951).
4. Samuels, L. T., *Recent Progress Hormone Res.*, **4**, 65 (1949).
5. Sweat, M. L., Samuels, L. T., and Lumry, R., *J. Biol. Chem.*, **185**, 75 (1950).
6. West, C. D., *Endocrinology*, **49**, 467 (1951).
7. West, C. D., Tyler, F. H., Brown, H., and Samuels, L. T., *J. Clin. Endocrinol.*, **11**, 897 (1951).
8. Lemon, H. M., Wotiz, H. H., and Robitscher, T., *J. Clin. Endocrinol. and Metabolism*, **13**, 948 (1953).
9. Wotiz, H. H., and Lemon, H. M., *J. Biol. Chem.*, **206**, 525 (1954).
10. Wotiz, H. H., Lemon, H. M., and Voulgaropoulos, A., *J. Biol. Chem.*, **209**, 437 (1954).
11. Richterich-van Baerle, R., Wotiz, H. H., and Lemon, H. M., *Experientia*, **10**, 308 (1954).
12. Clark, L. C., and Kochakian, C. D., *J. Biol. Chem.*, **170**, 23 (1947).
13. Levedahl, B. H., and Samuels, L. T., *J. Biol. Chem.*, **186**, 857 (1950).
14. Bischoff, F., and Pilhorn, H. R., *J. Biol. Chem.*, **174**, 663 (1948).
15. Eik-Nes, K., Schellman, J. A., Lumry, R., and Samuels, L. T., *J. Biol. Chem.*, **206**, 411 (1954).
16. Schellman, J. A., Lumry, R., and Samuels, L. T., *J. Am. Chem. Soc.*, **76**, 2808 (1954).

INDEX TO AUTHORS

A

Abelson, Philip H., and **Vogel, Henry J.** Amino acid biosynthesis in *Torulopsis utilis* and *Neurospora crassa*, 355

Anderson, E. P., Yen, C. Y., Mandel, H. George, and **Smith, Paul K.** Ureidosuccinic acid as a precursor of nucleic acid pyrimidines in normal and tumor-bearing mice, 625

Anderson, Roberta A. See *Hamilton and Anderson*, 249

Armstrong, Marvin D. The phenylalanine and tyrosine requirements of the rat, 409

— and **Shaw, Kenneth N. F.** Studies on phenylketonuria. III. The metabolism of *o*-tyrosine, 805

—, —, and **Robinson, Kathryn S.** Studies on phenylketonuria. II. The excretion of *o*-hydroxyphenylacetic acid in phenylketonuria, 797

Artom, Camillo. Effect of choline administration on the oxidation of fatty acids by extrahepatic tissues, 681

Ashmore, James. See *Renold, Hastings, Nesbett, and Ashmore*, 135

Asnis, Robert E. A glutathione reductase from *Escherichia coli*, 77

B

Baez, Silvio. See *Mazur, Baez, and Shorr*, 147

Baggett, Billy, Engel, Lewis L., and **Fielding, Linda L.** The estimation of hydroxysteroids, 87

Ball, Eric G. See *Strittmatter and Ball*, 445

Balls, A. K. See *Wood and Balls*, 297

Barnet, Harry N. See *Wick, Drury, Nakada, Barnet, and Morita*, 907

Basford, R. E. See *Huennekens, Basford, and Gabrio*, 951

Bernstein, W. See *Sinex, Plazin, Clareus, Bernstein, Van Slyke, and Chase*, 673

Black, Simon, and **Wright, Nancy G.** Aspartic β-semialdehyde dehydrogenase and aspartic β-semialdehyde, 39

— and —. β-Aspartokinase and β-aspartyl phosphate, 27

— and —. Homoserine dehydrogenase, 51

Bloch, Konrad. See *Snoke and Bloch*, 825

Bowser, Helen R. See *Magasanik and Bowser*, 571

Brand, Florence C. See *Sperry and Brand*, 69

Brown, Gene M., Ikawa, Miyoshi, and **Snell, Esmond E.** Synthesis and microbiological activity of some pantothenic acid conjugates, 855

—. See *Ward, Brown, and Snell*, 869

Brown, George Bosworth. See *Weinfeld, Roll, and Brown*, 523

Burstein, Shlomo, and **Dorfman, Ralph I.** Steroid metabolism in guinea pigs. I. Metabolism of cortisol *in vivo*, 581

—, —, and **Nadel, Eli M.** Corticosteroids in the urine of normal and scorbutic guinea pigs: isolation and quantitative determination, 597

C

Canellakis, E. S., and **Cohen, Philip P.** The end-products and intermediates of uric acid oxidation by uricase, 385

— and —. On the nature of oxonic acid and allantoxaidin as oxidation products of uric acid and allantoin, 379

—, **Tuttle, Alice L.,** and **Cohen, Philip P.** A comparative study of the end-products of uric acid oxidation by peroxidases, 397

Carter, Priscilla. See *Engel, Carter, and Fielding*, 99

Cathey, William J. See *Verly and Cathey*, 621

Cerecedo, Leopold R., and **Eich, Stephen.** Studies on thiamine analogues.

IV. Neopyrithiamine and oxyneopyrithiamine. A comparison of their effects *in vivo* and *in vitro*, 893

Chaikoff, I. L. See *Taurog, Potter, and Chaikoff*, 119

Chase, R. See *Sinex, Plazin, Clareus, Bernstein, Van Slyke, and Chase*, 673

Cheldelin, Vernon H. See *Wang, Christensen, and Cheldelin*, 365

Christensen, Bert E. See *Wang, Christensen, and Cheldelin*, 365

Clareus, D. See *Sinex, Plazin, Clareus, Bernstein, Van Slyke, and Chase*, 673

Cohen, Philip P. See *Canellakis and Cohen*, 379, 385

—. See *Canellakis, Tuttle, and Cohen*, 397

—. See *Lowenstein and Cohen*, 689

Cole, R. David, and **Li, Choh Hao.** Studies on pituitary lactogenic hormone. XIV. A simplified procedure of isolation, 197

Coon, Minor J. See *Rose, Leach, Coon, and Lambert*, 913

D

De Meio, R. H., Wizerkaniuk, Martha, and **Schreibman, I.** Enzymatic system synthesizing sulfuric acid esters of phenols, 439

Dimant, E., Landsberg, Edith, and **London, Irving M.** The metabolic behavior of reduced glutathione in human and avian erythrocytes, 769

Dixon, Jonathan S. See *Li, Geschwind, Dixon, Levy, and Harris*, 171

Dorfman, Ralph I. See *Burstein and Dorfman*, 581

—. See *Burstein, Dorfman, and Nadel*, 597

Drury, Douglas R. See *Wick, Drury, Nakada, Barnet, and Morita*, 907

E

Edelman, J., Ginsburg, V., and **Hassid, W. Z.** Conversion of monosaccharides to sucrose and cellulose in wheat seedlings, 843

Eich, Stephen. See *Cerecedo and Eich*, 893

Elwyn, David, Weissbach, Arthur, Henry, Sarah S., and **Sprinson, David B.** The biosynthesis of choline from serine and related compounds, 281

Engel, Lewis L., Carter, Priscilla, and **Fielding, Linda L.** Urinary metabolites of administered corticosterone. I. Steroids liberated by glucuronidase hydrolysis, 99

—. See *Baggett, Engel, and Fielding*, 87

Evans, Herbert M. See *Fels, Simpson, and Evans*, 311

F

Farmer, Chester J. See *Rupe and Farmer*, 899

Fels, I. Gordon, Simpson, Miriam E., and **Evans, Herbert M.** Purification of the anterior hypophyseal thyrotropic hormone, 311

Felton, S. See *Huennekens, Basford, and Gabrio*, 951

Fielding, Linda L. See *Baggett, Engel, and Fielding*, 87

—. See *Engel, Carter, and Fielding*, 99

Fish, William A. See *Stokes, Fish, and Hickey*, 325

Fishman, Rose. See *Wotiz, Richterich-van Baerle, and Lemon*, 969

G

Gabrio, Beverly Wescott. See *Huennekens, Basford, and Gabrio*, 951

Geschwind, Irving I. See *Levy, Geschwind, and Li*, 187

—. See *Li, Geschwind, Dixon, Levy, and Harris*, 171

Gilvarg, Charles. See *Yaniv and Gilvarg*, 787

Ginsburg, V. See *Edelman, Ginsburg, and Hassid*, 843

Gleason, Geoffrey I. Some notes on the exchange of iodine with thyroxine homologues, 837

Green, Maurice, and **Stahmann, Mark A.** Preparation and enzymatic hydrolysis

XUM

of poly-γ-ethylglutamyl bovine plasma albumin, 259
Greenberg, David M. See *Rabinovitz, Olson, and Greenberg,* 1

H

Hall, Leo M., and **MacVicar, Robert.** Ammonia as an intermediate in nitrate reduction by *Bacillus subtilis,* 305
Hamilton, Paul B., and **Anderson, Roberta A.** Hydroxylysine: isolation from gelatin and resolution of its diastereoisomers by ion exchange chromatography, 249
Harris, J. Ieuan, and **Li, Choh Hao.** Corticotropins (ACTH). IV. The action of carboxypeptidase on α-corticotropin, and the *C*-terminal amino acid sequence, 499
—. See *Li, Geschwind, Dixon, Levy, and Harris,* 171
Hassid, W. Z. See *Edelman, Ginsburg, and Hassid,* 843
Hastings, A. Baird. See *Renold, Hastings, Nesbett, and Ashmore,* 135
Henneman, Philip H., Rourke, G. Margaret, and **Jackson, W. P. U.** Depression of serum alkaline phosphatase activity by human serum albumin, 19
Henry, Sarah S. See *Elwyn, Weissbach, Henry, and Sprinson,* 281
Herbert, Edward, Potter, Van R., and **Takagi, Yasuyuki.** Nucleotide metabolism. IV. The phosphorylation of 5′-uridine nucleotides by cell fractions from rat liver, 923
Hickey, Frederick C. See *Stokes, Fish, and Hickey,* 325
Hofmann, Klaus, and **Tausig, Fred.** The chemical nature of the fatty acids of a group C *Streptococcus* species, 415
— and —. On the identity of phytomonic and lactobacillic acids. A reinvestigation of the fatty acid spectrum of *Agrobacterium* (*Phytomonas*) *tumefaciens,* 425
Horner, William H. See *Melville, Horner, Otken, and Ludwig,* 61
Huennekens, F. M., Basford, R. E., and **Gabrio, Beverly Wescott.** An oxidase for reduced diphosphopyridine nucleotide, 951
Huffman, Max N., and **Lott, Mary Harriet.** 16-Substituted steroids. XI. Estradiol-3,16β, 343
Humphreys, T. E., and **Stumpf, P. K.** Fat metabolism in higher plants. IV. Preparation of soluble fatty acid oxidases from peanut microsomes, 941

I

Ikawa, Miyoshi, and **O'Barr, Jean S.** The isolation of α,α′-diaminopimelic acid from *Lactobacillus arabinosus,* 877
—. See *Brown, Ikawa, and Snell,* 855

J

Jackson, W. P. U. See *Henneman, Rourke, and Jackson,* 19
Jedeikin, Lillian A., and **Weinhouse, Sidney.** Metabolism of neoplastic tissue. VI. Assay of oxidized and reduced diphosphopyridine nucleotide in normal and neoplastic tissues, 271

K

Kovachevich, R., and **Wood, W. A.** Carbohydrate metabolism by *Pseudomonas fluorescens.* III. Purification and properties of a 6-phosphogluconate dehydrase, 745
— and —. IV. Purification and properties of 2-keto-3-deoxy-6-phosphogluconate aldolase, 757

L

Lambert, G. Frederick. See *Rose, Leach, Coon, and Lambert,* 913
Landsberg, Edith. See *Dimant, Landsberg, and London,* 769
Langner, Ralph R., and **Volkmann, Carol M.** The fate of ingested acetyl-L- and D-tryptophan in the human subject, 433
Laskowski, M. See *Wu and Laskowski,* 609

Leach, Byron E. See *Rose, Leach, Coon, and Lambert,* 913
Lemon, Henry M. See *Wotiz, Richterich-van Baerle, and Lemon,* 969
Levy, Anthony L., Geschwind, Irving I., and **Li, Choh Hao.** Corticotropins (ACTH). II. Amino acid composition of α-corticotropin, 187
— and **Li, Choh Hao.** Corticotropins (ACTH). III. Kinetic studies on the reaction of *N*-terminal group reagents with α-corticotropin, and *N*-terminal group analysis, 487
—. See *Li, Geschwind, Dixon, Levy, and Harris,* 171
Levy, Hilton B. See *Webb and Levy,* 107
Li, Choh Hao, Geschwind, Irving I., Dixon, Jonathan S., Levy, Anthony L., and **Harris, J. Ieuan.** Corticotropins (ACTH). I. Isolation of α-corticotropin from sheep pituitary glands, 171
—. See *Cole and Li,* 197
—. See *Harris and Li,* 499
—. See *Levy, Geschwind, and Li,* 187
—. See *Levy and Li,* 487
Li, Yao Wen. See *Shepherd, Li, Mason, and Ziffren,* 405
Linker, Alfred, Meyer, Karl, and **Weissmann, Bernard.** Enzymatic formation of monosaccharides from hyaluronate, 237
Loewus, Frank A. See *Tchen, Loewus, and Vennesland,* 547
London, Irving M. See *Dimant, Landsberg, and London,* 769
Longenecker, J. B., and **Snell, Esmond E.** A possible mechanism for kynureninase action, 229
Lott, Mary Harriet. See *Huffman and Lott,* 343
Lowenstein, J. M., and **Cohen, Philip P.** Studies on the mechanism of carbamylaspartic acid synthesis, 689
Lowry, Oliver H. See *Strominger and Lowry,* 635
Ludwig, Martha L. See *Melville, Horner, Otken, and Ludwig,* 61

M

MacVicar, Robert. See *Hall and MacVicar,* 305
Magasanik, Boris. The metabolic control of histidine assimilation and dissimilation in *Aerobacter aerogenes,* 557
— and **Bowser, Helen R.** The degradation of histidine by *Aerobacter aerogenes,* 571
Mandel, H. George. See *Anderson, Yen, Mandel, and Smith,* 625
Mann, George V. See *Portman and Mann,* 733
Marx, Walter. See *Weiss and Marx,* 349
Mason, Edward E. See *Shepherd, Li, Mason, and Ziffren,* 405
Mazur, Abraham, Baez, Silvio, and **Shorr, Ephraim.** The mechanism of iron release from ferritin as related to its biological properties, 147
Meinhart, Josephine O., and **Simmonds, Sofia.** Serine metabolism in a mutant strain of *Escherichia coli* strain K-12, 329
Melville, Donald B., Horner, William H., Otken, Charles C., and **Ludwig, Martha L.** Studies on the origin of ergothioneine in animals, 61
Meyer, Karl. See *Linker, Meyer, and Weissmann,* 237
Morita, Toshiko N. See *Wick, Drury, Nakada, Barnet, and Morita,* 907
Morrison, Martin, and **Stotz, Elmer.** Partition chromatography of hemins. Separation of the prosthetic groups of cytochromes *a* and a_3, 373
Mortenson, L. E., and **Wilson, P. W.** Metabolism of ribose-5-phosphate by *Azotobacter vinelandii,* 713

N

Nadel, Eli M. See *Burstein, Dorfman, and Nadel,* 597
Nakada, Henry I. See *Wick, Drury, Nakada, Barnet, and Morita,* 907
Naschke, Mary Dell. See *Seaman and Naschke,* 705

Nason, Alvin. See *Zucker and Nason*, 463

Nesbett, Frances B. See *Renold, Hastings, Nesbett, and Ashmore*, 135

Nichol, Charles A. See *Zakrzewski and Nichol*, 697

Nurk, E. See *Huennekens, Basford, and Gabrio*, 951

O

O'Barr, Jean S. See *Ikawa and O'Barr*, 877

Olivard, Joanne, and **Snell, Esmond E.** Growth and enzymatic activities of vitamin B_6 analogues. I. D-Alanine synthesis, 203

— and —. II. Synthesis of miscellaneous amino acids, 215

Olson, Margaret E. See *Rabinovitz, Olson, and Greenberg*, 1

Otken, Charles C. See *Melville, Horner, Otken, and Ludwig*, 61

P

Plazin, John. See *Sinex, Plazin, Clareus, Bernstein, Van Slyke, and Chase*, 673

Portman, Oscar W., and **Mann, George V.** The disposition of taurine-S^{35} and taurocholate-S^{35} in the rat: dietary influences, 733

Potter, G. D. See *Taurog, Potter, and Chaikoff*, 119

Potter, Van R. See *Herbert, Potter, and Takagi*, 923

Poujol, Jean. See *Rivoire, Rivoire, and Poujol*, 11

R

Rabinovitz, M., Olson, Margaret E., and **Greenberg, David M.** Relation of energy processes to the incorporation of amino acids into proteins of the Ehrlich ascites carcinoma, 1

Rafelson, Max E., Jr. Conversion of radioactive glucose and acetate to tryptophan by *Aerobacter aerogenes*, 479

Renold, Albert E., Hastings, A. Baird, Nesbett, Frances B., and **Ashmore, James.** Studies on carbohydrate metabolism in rat liver slices. IV. Biochemical sequence of events after insulin administration, 135

Richterich-van Baerle, Roland. See *Wotiz, Richterich-van Baerle, and Lemon*, 969

Rivoire, Jane. See *Rivoire, Rivoire, and Poujol*, 11

Rivoire, Raymond, Rivoire, Jane, and **Poujol, Jean.** The determination of urinary 17-trioxysteroids, 11

Robinson, Kathryn S. See *Armstrong, Shaw, and Robinson*, 797

Rodkey, F. Lee. Oxidation-reduction potentials of the diphosphopyridine nucleotide system, 777

Roll, Paul M., and **Weliky, Irving.** The utilization of nucleotides by the mammal. II. N^{15}-labeled purine nucleotides, 509

—. See *Weinfeld, Roll, and Brown*, 523

Rose, William C., Leach, Byron E., Coon, Minor J., and **Lambert, G. Frederick.** The amino acid requirements of man. IX. The phenylalanine requirement, 913

Rourke, G. Margaret. See *Henneman, Rourke, and Jackson*, 19

Rupe, C. O., and **Farmer, Chester J.** Amino acid studies in the transformation of proteins of the hen's egg to tissue proteins during incubation, 899

S

Schreibman, I. See *De Meio, Wizerkaniuk, and Schreibman*, 439

Seaman, Gerald R., and **Naschke, Mary Dell.** Removal of thioctic acid from enzymes, 705

Segal, Harold L. Kinetic studies on the phenol-sulfate conjugating system of rat liver, 161

Shaw, Kenneth N. F. See *Armstrong and Shaw*, 805

—. See *Armstrong, Shaw, and Robinson*, 797

Shepherd, James A., Li, Yao Wen, Mason, Edward E., and **Ziffren, Sidney E.** The distribution of aconitase and fumarase in homogenates of human liver, 405
Sherry, Sol. See *Troll and Sherry*, 881
Shorr, Ephraim. See *Mazur, Baez, and Shorr*, 147
Shulman, N. Raphael. A proteolytic inhibitor with anticoagulant activity separated from human urine and plasma, 655
Siegelman, H. W. Quercetin glycosides of Grimes Golden apple skin, 647
Simmonds, Sofia. See *Meinhart and Simmonds*, 329
Simpson, Miriam E. See *Fels, Simpson, and Evans*, 311
Sinex, F. Marott, Plazin, John, Clareus, D., Bernstein, W., Van Slyke, Donald D., and **Chase, R.** Determination of total carbon and its radioactivity. II. Reduction of required voltage and other modifications, 673
Skogstrom, Paul R. See *Burstein and Dorfman*, 581
Smith, Paul K. See *Anderson, Yen, Mandel, and Smith*, 625
Snell, Esmond E. See *Brown, Ikawa, and Snell*, 855
—. See *Longenecker and Snell*, 229
—. See *Olivard and Snell*, 203, 215
—. See *Ward, Brown, and Snell*, 869
Snoke, John E. Isolation and properties of yeast glutathione synthetase, 813
— and **Bloch, Konrad.** Studies on the mechanism of action of glutathione synthetase, 825
Sperry, Warren M., and **Brand, Florence C.** The determination of total lipides in blood serum, 69
Sprinson, David B. See *Elwyn, Weissbach, Henry, and Sprinson*, 281
Stahmann, Mark A. See *Green and Stahmann*, 259
Stetten, DeWitt, Jr. See *Stetten and Stetten*, 723
Stetten, Marjorie R., and **Stetten, DeWitt, Jr.** Glycogen regeneration *in vivo*, 723
Stokes, William M., Fish, William A., and **Hickey, Frederick C.** The fate of injected cholestenone in the intact rat, 325
Stotz, Elmer. See *Morrison and Stotz*, 373
Strittmatter, Philipp, and **Ball, Eric G.** Formaldehyde dehydrogenase, a glutathione-dependent enzyme system, 445
Strominger, Jack L., and **Lowry, Oliver H.** The quantitative histochemistry of brain. IV. Lactic, malic, and glutamic dehydrogenases, 635
Stumpf, P. K. See *Humphreys and Stumpf*, 941

T

Takagi, Yasuyuki. See *Herbert, Potter, and Takagi*, 923
Taurog, Alvin, Potter, G. D., and **Chaikoff, I. L.** Conversion of inorganic I^{131} to organic I^{131} by cell-free preparations of thyroid tissue, 119
Tausig, Fred. See *Hofmann and Tausig*, 415, 425
Tchen, T. T., Loewus, Frank A., and **Vennesland, Birgit.** The mechanism of enzymatic carbon dioxide fixation into oxalacetate, 547
— and **Vennesland, Birgit.** Enzymatic carbon dioxide fixation into oxalacetate in wheat germ, 533
Tillotson, Arline M. See *Burstein and Dorfman*, 581
Troll, Walter, and **Sherry, Sol.** The activation of human plasminogen by streptokinase, 881
Tuttle, Alice L. See *Canellakis, Tuttle, and Cohen*, 397

V

Van Slyke, Donald D. See *Sinex, Plazin, Clareus, Bernstein, Van Slyke, and Chase*, 673
Vennesland, Birgit. See *Tchen, Loewus, and Vennesland*, 547
—. See *Tchen and Vennesland*, 533
Verly, Walter G., and **Cathey, William J.** The influence of vitamin B_{12} on

the biosynthesis of the methyl group of choline from methanol, 621
Vogel, Henry J. See *Abelson and Vogel*, 355
Volkmann, Carol M. See *Langner and Volkmann*, 433

W

Wang, Chih H., Christensen, Bert E., and **Cheldelin, Vernon H.** Conversion of pyruvic acid-2-C^{14} to some aliphatic monoamino acids in yeast, 365
Ward, G. B., Brown, Gene M., and **Snell, Esmond E.** Phosphorylation of pantothenic acid and pantethine by an enzyme from *Proteus morganii*, 869
Webb, Junius M., and **Levy, Hilton B.** A sensitive method for the determination of deoxyribonucleic acid in tissues and microorganisms, 107
Weinfeld, Herbert, Roll, Paul M., and **Brown, George Bosworth.** The utilization of nucleotides by the mammal. III. Isomeric purine nucleotides labeled with C^{14}, 523
Weinhouse, Sidney. See *Jedeikin and Weinhouse*, 271
Weiss, Samuel B., and **Marx, Walter.** The fate of radioactive cholesterol in mice with modified thyroid activities, 349
Weissbach, Arthur. See *Elwyn, Weissbach, Henry, and Sprinson*, 281
Weissman, Bernard. See *Linker, Meyer, and Weissman*, 237
Weliky, Irving. See *Roll and Weliky*, 509
Wick, Arne N., Drury, Douglas R., Nakada, Henry I., Barnet, Harry N., and **Morita, Toshiko N.** Glucosamine and the action of insulin, 907
Wilson, P. W. See *Mortenson and Wilson*, 713
Wizerkaniuk, Martha. See *De Meio, Wizerkaniuk, and Schreibman*, 439
Wood, Henry N., and **Balls, A. K.** Enzymatic oxidation of α-chymotrypsin, 297
Wood, W. A. See *Kovachevich and Wood*, 745, 757
Wotiz, Herbert H., Richterich-van Baerle, Roland, and **Lemon, Henry M.** Studies in steroid metabolism. III. Metabolism of testosterone by human serum, 969
Wright, Nancy G. See *Black and Wright*, 27, 39, 51
Wu, Feng Chi, and **Laskowski, M.** Action of the naturally occurring trypsin inhibitors against chymotrypsins α and B, 609

Y

Yaniv, Haim, and **Gilvarg, Charles.** Aromatic biosynthesis. XIV. 5-Dehydroshikimic reductase, 787
Yen, C. Y. See *Anderson, Yen, Mandel, and Smith*, 625

Z

Zakrzewski, Sigmund F., and **Nichol, Charles A.** The incorporation of formate-C^{14} into *citrovorum* factor, 697
Ziffren, Sidney E. See *Shepherd, Li, Mason, and Ziffren*, 405
Zucker, Milton, and **Nason, Alvin.** A pyridine nucleotide-hydroxylamine reductase from *Neurospora*, 463

A

A

A

A

A

A

A

A

A

A

A

A

A

A

A

INDEX TO SUBJECTS

A

Acetate: Oxal-. *See* Oxalacetate
Radioactive, conversion to tryptophan, *Aerobacter aerogenes, Rafelson,* 479
Acetic acid: *o*-Hydroxyphenyl-. *See* Hydroxyphenylacetic acid
Acetyl-D-tryptophan: Fate, *Langner and Volkmann,* 433
Acetyl-L-tryptophan: Fate, *Langner and Volkmann,* 433
Aconitase: Liver, *Shepherd, Li, Mason, and Ziffren,* 405
Aerobacter aerogenes: Acetate, radioactive, conversion to tryptophan, *Rafelson,* 479
Glucose, radioactive, conversion to tryptophan, *Rafelson,* 479
Histidine assimilation and dissimilation, metabolic control, *Magasanik,* 557
— degradation, *Magasanik and Bowser,* 571
Agrobacterium tumefaciens: Fatty acids, *Hofmann and Tausig,* 425
Alanine: D-, synthesis, *Olivard and Snell,* 203
Albumin: Blood serum, blood serum phosphatase, alkaline, effect, *Henneman, Rourke, and Jackson,* 19
Poly-γ-ethylglutamyl. *See* Poly-γ-ethylglutamyl albumin
Aldolase: 2-Keto-3-deoxy-6-phosphogluconate. *See* Keto-3-deoxy-6-phosphogluconate aldolase
Allantoin: Oxidation, oxonic acid and allantoxaidin relation, *Canellakis and Cohen,* 379
Allantoxaidin: Uric acid and allantoin oxidation, relation, *Canellakis and Cohen,* 379
Amino acid(s): Biosynthesis, *Neurospora crassa, Abelson and Vogel,* 355
—, *Torulopsis utilis, Abelson and Vogel,* 355
Amino acid(s)—*continued:*
α-Corticotropin, *Levy, Geschwind, and Li,* 187
Egg proteins to tissue proteins, relation, *Rupe and Farmer,* 899
Mono-. *See* Monoamino acid
Protein incorporation from, Ehrlich ascites carcinoma, energy processes, relation, *Rabinovitz, Olson, and Greenberg,* 1
Requirements, *Rose, Leach, Coon, and Lambert,* 913
Synthesis, *Olivard and Snell,* 215
Ammonia: Nitrate reduction, *Bacillus subtilis,* relation, *Hall and MacVicar,* 305
Anticoagulant: Proteolytic inhibitor, urine and blood plasma, *Shulman,* 655
Apple: Skin, quercetin glycosides, *Siegelman,* 647
Ascites: Carcinoma, Ehrlich, amino acid incorporation into protein, energy processes, relation, *Rabinovitz, Olson, and Greenberg,* 1
Aspartic acid: Carbamyl-. *See* Carbamylaspartic acid
Aspartic β-semialdehyde: *Black and Wright,* 39
Aspartic β-semialdehyde dehydrogenase: *Black and Wright,* 39
Aspartokinase: β-, *Black and Wright,* 27
Aspartyl phosphate: β-, *Black and Wright,* 27
Azotobacter vinelandii: Ribose-5-phosphate metabolism, *Mortenson and Wilson,* 713

B

Bacillus: *See also Lactobacillus*
Bacillus subtilis: Nitrate reduction, ammonia relation, *Hall and MacVicar,* 305

Bacteria: *See also Aerobacter, Agrobacterium, Azotobacter, Escherichia, Leuconostoc, Proteus, Pseudomonas, Streptococcus*
Biosynthesis: Aromatic, 5-dehydroshikimic reductase relation, *Yaniv and Gilvarg*, 787
Blood cell(s): Red, avian, glutathione, reduced, metabolism, *Dimant, Landsberg, and London*, 769
Blood plasma: Poly-γ-ethylglutamyl albumin, preparation and hydrolysis, enzymatic, *Green and Stahmann*, 259
Proteolytic inhibitor, anticoagulant properties, *Shulman*, 655
Blood serum: Albumin, blood serum phosphatase, alkaline, effect, *Henneman, Rourke, and Jackson*, 19
Lipides, total, determination, *Sperry and Brand*, 69
Phosphatase, alkaline, blood serum albumin effect, *Henneman, Rourke, and Jackson*, 19
Testosterone metabolism, effect, *Wotiz, Richterich-van Baerle, and Lemon*, 969
Brain: Glutamic dehydrogenase, determination, *Strominger and Lowry*, 635
Histochemistry, *Strominger and Lowry*, 635
Lactic dehydrogenase, determination, *Strominger and Lowry*, 635
Malic dehydrogenase, determination, *Strominger and Lowry*, 635

C

Carbamylaspartic acid: Synthesis mechanism, *Lowenstein and Cohen*, 689
Carbohydrate: Metabolism, liver, insulin effect, *Renold, Hastings, Nesbett, and Ashmore*, 135
—, *Pseudomonas fluorescens, Kovachevich and Wood*, 745, 757
Carbon: Total, determination, *Sinex, Plazin, Clareus, Bernstein, Van Slyke, and Chase*, 673
—, radioactivity, determination, *Sinex, Plazin, Clareus, Bernstein, Van Slyke, and Chase*, 673
Carbon dioxide: Fixation into oxalacetate, enzymatic, mechanism, *Tchen, Loewus, and Vennesland*, 547
— — —, —, wheat germ, *Tchen and Vennesland*, 533
Carboxypeptidase: α-Corticotropin, effect, *Harris and Li*, 499
Carcinoma: Ascites, Ehrlich, amino acid incorporation into protein, energy processes, relation, *Rabinovitz, Olson, and Greenberg*, 1
See also Neoplasm, Tumor
Cellulose: Monosaccharide conversion to, wheat seedlings, *Edelman, Ginsburg, and Hassid*, 843
Cholestenone: Fate, *Stokes, Fish, and Hickey*, 325
Cholesterol: Radioactive, fate, thyroid effect, *Weiss and Marx*, 349
Choline: Biosynthesis from serine, *Elwyn, Weissbach, Henry, and Sprinson*, 281
Fatty acid oxidation, effect, *Artom*, 681
Methyl group biosynthesis from methanol, vitamin B_{12} effect, *Verly and Cathey*, 621
Chymotrypsin(s): α-, oxidation, enzymatic, *Wood and Balls*, 297
α, trypsin inhibitors, action, *Wu and Laskowski*, 609
B, trypsin inhibitors, action, *Wu and Laskowski*, 609
Citrovorum factor: *See Leuconostoc citrovorum*
Coagulation: *See also* Anticoagulant
Corticosteroid(s): Urine, isolation and determination, scurvy, *Burstein, Dorfman, and Nadel*, 597
Corticosterone: Metabolites, urine, *Engel, Carter, and Fielding*, 99
Corticotropin(s): *Li, Geschwind, Dixon, Levy, and Harris*, 171
Levy, Geschwind, and Li, 187
Harris and Li, 499
Levy and Li, 487
α-, amino acids, *Levy, Geschwind, and Li*, 187
—, carboxypeptidase action, *Harris and Li*, 499

Corticotropin(s)—*continued:*
α-, isolation, pituitary, *Li, Geschwind, Dixon, Levy, and Harris,* 171
—, nitrogen-terminal group reagents, reaction, *Levy and Li,* 487
Cortisol: Metabolism, *Burstein and Dorfman,* 581
Cytochrome: *a*, prosthetic groups, *Morrison and Stotz,* 373
a_3, prosthetic groups, *Morrison and Stotz,* 373

D

Dehydrase: 6-Phosphogluconate. *See* Phosphogluconate dehydrase
Dehydrogenase: Aspartic β-semialdehyde. *See* Aspartic β-semialdehyde dehydrogenase
Formaldehyde. *See* Formaldehyde dehydrogenase
Glutamic. *See* Glutamic dehydrogenase
Homoserine. *See* Homoserine dehydrogenase
Lactic. *See* Lactic dehydrogenase
Malic. *See* Malic dehydrogenase
Dehydroshikimic reductase: 5-, aromatic biosynthesis, relation, *Yaniv and Gilvarg,* 787
Deoxyribonucleic acid: Microorganisms, determination, *Webb and Levy,* 107
Tissues, determination, *Webb and Levy,* 107
Diaminopimelic acid: α,α′-, isolation, *Lactobacillus arabinosus, Ikawa and O'Barr,* 877
Diet: Taurine, sulfur 35-labeled, metabolism, effect, *Portman and Mann,* 733
Taurocholate, sulfur 35-labeled, metabolism, effect, *Portman and Mann,* 733
Diphosphopyridine nucleotide: Neoplasm, determination, *Jedeikin and Weinhouse,* 271
Reduced, oxidase effect, *Huennekens, Basford, and Gabrio,* 951
System, oxidation-reduction potentials, *Rodkey,* 777

E

Egg: Proteins, tissue proteins from, amino acids, relation, *Rupe and Farmer,* 899
Ehrlich: Ascites carcinoma, amino acid incorporation into protein, energy processes, relation, *Rabinovitz, Olson, and Greenberg,* 1
Energy: Processes, amino acid incorporation into protein, Ehrlich ascites carcinoma, relation, *Rabinovitz, Olson, and Greenberg,* 1
Enzyme(s): Blood plasma poly-γ-ethylglutamyl albumin, hydrolysis, *Green and Stahmann,* 259
Carbon dioxide fixation into oxalacetate, mechanism, *Tchen, Loewus, and Vennesland,* 547
— — — — oxalacetate, wheat germ, *Tchen and Vennesland,* 533
α-Chymotrypsin oxidation, *Wood and Balls,* 297
Monosaccharide formation from hyaluronate, *Linker, Meyer, and Weissmann,* 237
Proteus morganii, pantothenic acid and pantethine phosphorylation, *Ward, Brown, and Snell,* 869
System, phenol sulfuric acid esters, synthesis, effect, *De Meio, Wizerkaniuk, and Schreibman,* 439
Thioctic acid removal, *Seaman and Naschke,* 705
Vitamin B_6 analogues, action, *Olivard and Snell,* 203, 215
See also Aconitase, Chymotrypsin, etc.
Ergothioneine: Origin, *in vivo, Melville, Horner, Otken, and Ludwig,* 61
Erythrocyte(s): *See* Blood cell, red
Escherichia coli: Glutathione reductase, *Asnis,* 77
Mutant, serine metabolism, *Meinhart and Simmonds,* 329
Estradiol: *Huffman and Lott,* 343

F

Fat: Metabolism, plants, *Humphreys and Stumpf,* 941
Fatty acid(s): *Agrobacterium tumefaciens, Hofmann and Tausig,* 425

Fatty acid(s)—*continued:*
Oxidation, choline effect, *Artom*, 681
Streptococcus, *Hofmann and Tausig*, 415
Fatty acid oxidase(s): Peanut microsomes, preparation, *Humphreys and Stumpf*, 941
Ferritin: Iron release, *Mazur, Baez, and Shorr*, 147
Formaldehyde dehydrogenase: Glutathione relation, *Strittmatter and Ball*, 445
Formate: Carbon 14-labeled, incorporation into citrovorum factor, *Zakrzewski and Nichol*, 697
Fumarase: Liver, *Shepherd, Li, Mason, and Ziffren*, 405

G

Gelatin: Hydroxylysine isolation, *Hamilton and Anderson*, 249
Glucosamine: Insulin action and, *Wick, Drury, Nakada, Barnet, and Morita*, 907
Glucose: Radioactive, conversion to tryptophan, *Aerobacter aerogenes*, *Rafelson*, 479
Glucuronidase: Urine steroids, hydrolysis, *Engel, Carter, and Fielding*, 99
Glutamic dehydrogenase: Brain, determination, *Strominger and Lowry*, 635
Glutathione: Formaldehyde dehydrogenase, relation, *Strittmatter and Ball*, 445
Reduced, metabolism, blood cells, red, avian, *Dimant, Landsberg, and London*, 769
Glutathione reductase: *Escherichia coli*, *Asnis*, 77
Glutathione synthetase: Action, mechanism, *Snoke and Bloch*, 825
Yeast, isolation and properties, *Snoke*, 813
Glycogen: Regeneration *in vivo*, *Stetten and Stetten*, 723
Glycoside(s): Quercetin, apple skin, *Siegelman*, 647
Growth: Vitamin B_6 analogues, effect, *Olivard and Snell*, 203, 215

H

Hemin(s): Chromatography, partition, *Morrison and Stotz*, 373
Histidine: Assimilation and dissimilation, *Aerobacter aerogenes*, metabolic control, *Magasanik*, 557
Degradation, *Aerobacter aerogenes*, *Magasanik and Bowser*, 571
Homoserine dehydrogenase: *Black and Wright*, 51
Hormone: Thyrotropic. *See* Thyrotropic hormone
Hyaluronate: Monosaccharide formation from, enzymatic, *Linker, Meyer, and Weissmann*, 237
Hydroxylysine: Diastereoisomers, *Hamilton and Anderson*, 249
Isolation, gelatin, *Hamilton and Anderson*. 249
Hydroxyphenylacetic acid: *o*-, excretion, phenylketonuria, *Armstrong, Shaw, and Robinson*, 797
Hydroxysteroid(s): Determination, *Baggett, Engel, and Fielding*, 87
Hypophysis: *See* Pituitary

I

Insulin: Carbohydrate metabolism, liver, effect, *Renold, Hastings, Nesbett, and Ashmore*, 135
Glucosamine effect, *Wick, Drury, Nakada, Barnet, and Morita*, 907
Iodine: Exchange, thyroxine homologues, *Gleason*, 837
Inorganic, labeled, conversion to iodine, organic, labeled, thyroid effect, *Taurog, Potter, and Chaikoff*, 119
Iron: Release, ferritin, *Mazur, Baez, and Shorr*, 147

K

Keto-3-deoxy-6-phosphogluconate aldolase: 2-, *Pseudomonas fluorescens*, purification and properties, *Kovachevich and Wood*, 757
Ketonuria: Phenyl-. *See* Phenylketonuria
Kinase: β-Asparto-. *See* Aspartokinase
Strepto-. *See* Streptokinase

Kynureninase: Action, mechanism, *Longenecker and Snell*, 229

L

Lactic dehydrogenase: Brain, determination, *Strominger and Lowry*, 635
Lactobacillic acid: *Hofmann and Tausig*, 425
Lactobacillus arabinosus: α,α'-Diaminopimelic acid isolation, *Ikawa and O'Barr*, 877
Lactogenic hormone: Isolation, pituitary, *Cole and Li*, 197
Leuconostoc citrovorum: Factor, formate, carbon 14-labeled, incorporation into, *Zakrzewski and Nichol*, 697
Lipide(s): Total, blood serum, determination, *Sperry and Brand*, 69
Liver: Aconitase, *Shepherd, Li, Mason, and Ziffren*, 405
Carbohydrate metabolism, insulin effect, *Renold, Hastings, Nesbett, and Ashmore*, 135
Fumarase, *Shepherd, Li, Mason, and Ziffren*, 405
Phenol-sulfate conjugating system, *Segal*, 161
5′-Uridine nucleotide phosphorylation, *Herbert, Potter, and Takagi*, 923
Lysine: Hydroxy-. *See* Hydroxylysine

M

Malic dehydrogenase: Brain, determination, *Strominger and Lowry*, 635
Methanol: Choline methyl group biosynthesis from, vitamin B_{12} effect, *Verly and Cathey*, 621
Microorganism(s): Deoxyribonucleic acid, determination, *Webb and Levy*, 107
See also Bacillus, Bacteria
Mold: *See also Neurospora*
Monoamino acid(s): Pyruvic acid, carbon 14-labeled, conversion to, yeast, *Wang, Christensen, and Cheldelin*, 365
Monosaccharide(s): Formation, enzymatic, from hyaluronate, *Linker, Meyer, and Weissmann*, 237
Sucrose and cellulose from, wheat seedlings, *Edelman, Ginsburg, and Hassid*, 843

N

Neoplasm: Diphosphopyridine nucleotide, determination, *Jedeikin and Weinhouse*, 271
Metabolism, *Jedeikin and Weinhouse*, 271
See also Carcinoma, Tumor
Neopyrithiamine: *Cerecedo and Eich*, 893
Neurospora crassa: Amino acid biosynthesis, *Abelson and Vogel*, 355
Pyridine nucleotide-hydroxylamine reductase, *Zucker and Nason*, 463
Nitrate: Reduction, *Bacillus subtilis*, ammonia relation, *Hall and MacVicar*, 305
Nitrogen: -Terminal group analysis, *Levy and Li*, 487
Nucleic acid: Deoxyribo-. *See* Deoxyribonucleic acid
Pyrimidines, ureidosuccinic acid as precursor, tumor-bearing mice, *Anderson, Yen, Mandel, and Smith*, 625
Nucleotide(s): Diphosphopyridine. *See* Diphosphopyridine nucleotide
Metabolism, *Herbert, Potter, and Takagi*, 923
Purine. *See* Purine nucleotide
5′-Uridine. *See* Uridine nucleotide
Utilization, *Roll and Weliky*, 509
Weinfeld, Roll, and Brown, 523

O

Oxalacetate: Carbon dioxide fixation into, enzymatic, mechanism, *Tchen, Loewus, and Vennesland*, 547
— — — —, enzymatic, wheat germ, *Tchen and Vennesland*, 533
Oxidase(s): Diphosphopyridine nucleotide, reduced, effect, *Huennekens, Basford, and Gabrio*, 951
Fatty acid. *See* Fatty acid oxidase

Oxonic acid: Uric acid and allantoin oxidation, relation, *Canellakis and Cohen*, 379
Oxyneopyrithiamine: *Cerecedo and Eich*, 893

P

Pantethine: Phosphorylation, *Proteus morganii* enzyme, *Ward, Brown, and Snell*, 869
Pantothenic acid: Conjugates, synthesis and microbiological activity, *Brown, Ikawa, and Snell*, 855
Phosphorylation, *Proteus morganii* enzyme, *Ward, Brown, and Snell*, 869
Peanut: Microsomes, fatty acid oxidases, preparation, *Humphreys and Stumpf*, 941
Peptidase: Carboxy-. *See* Carboxypeptidase
Peroxidase(s): Uric acid oxidation, effect, *Canellakis, Tuttle, and Cohen*, 397
Phenol: -Sulfate conjugating system, liver, *Segal*, 161
Sulfuric acid esters, synthesis, enzyme system effect, *De Meio, Wizerkaniuk, and Schreibman*, 439
Phenylalanine: Requirements, *Armstrong*, 409
Rose, Leach, Coon, and Lambert, 913
Phenylketonuria: *o*-Hydroxyphenylacetic acid excretion, *Armstrong, Shaw, and Robinson*, 797
o-Tyrosine metabolism, *Armstrong and Shaw*, 805
Phosphatase: Alkaline, blood serum, blood serum albumin effect, *Henneman, Rourke, and Jackson*, 19
Phosphogluconate dehydrase: 6-, *Pseudomonas fluorescens*, purification and properties, *Kovachevich and Wood*, 745
Phytomonas tumefaciens: *See also Agrobacterium tumefaciens*
Phytomonic acid: *Hofmann and Tausig*, 425
Pimelic acid: α,α'-Diamino-. *See* Diaminopimelic acid
Pituitary: Anterior, thyrotropic hormone, purification, *Fels, Simpson, and Evans*, 311
α-Corticotropin isolation, *Li, Geschwind, Dixon, Levy, and Harris*, 171
Lactogenic hormone isolation, *Cole and Li*, 197
Plant(s): Fat metabolism, *Humphreys and Stumpf*, 941
Plasminogen: Streptokinase effect, *Troll and Sherry*, 881
Poly-γ-ethylglutamyl albumin: Blood plasma, preparation and hydrolysis, enzymatic, *Green and Stahmann*, 259
Protein(s): Amino acid incorporation into, Ehrlich ascites carcinoma, energy processes, relation, *Rabinovitz, Olson, and Greenberg*, 1
Egg, tissue protein from, amino acids, relation, *Rupe and Farmer*, 899
Proteolysis: Inhibitor, anticoagulant properties, urine and blood plasma, *Shulman*, 655
Proteus morganii: Enzyme, pantothenic acid and pantethine phosphorylation, *Ward, Brown, and Snell*, 869
Pseudomonas fluorescens: Carbohydrate metabolism, *Kovachevich and Wood*, 745, 757
2-Keto-3-deoxy-6-phosphogluconate aldolase, purification and properties, *Kovachevich and Wood*, 757
6-Phosphogluconate dehydrase, purification and properties, *Kovachevich and Wood*, 745
Purine nucleotide(s): Isomeric, carbon 14-labeled, utilization, *Weinfeld, Roll, and Brown*, 523
Nitrogen 15-labeled, utilization, *Roll and Weliky*, 509
Pyridine nucleotide-hydroxylamine reductase: *Neurospora, Zucker and Nason*, 463
Pyrimidine(s): Nucleic acid, ureidosuccinic acid as precursor, tumor-bearing mice, *Anderson, Yen, Mandel, and Smith*, 625

Pyruvic acid: Carbon 14-labeled, conversion to monoamino acids, yeast, *Wang, Christensen, and Cheldelin*, 365

Q

Quercetin: Glycosides, apple skin, *Siegelman*, 647

R

Reductase: 5-Dehydroshikimic. *See* Dehydroshikimic reductase
Glutathione. *See* Glutathione reductase
Pyridine nucleotide-hydroxylamine. *See* Pyridine nucleotide-hydroxylamine reductase
Ribose-5-phosphate: Metabolism, *Azotobacter vinelandii, Mortenson and Wilson*, 713

S

Scurvy: Urine corticosteroids, isolation and determination, *Burstein, Dorfman, and Nadel*, 597
Serine: Choline biosynthesis from, *Elwyn, Weissbach, Henry, and Sprinson*, 281
Metabolism, *Escherichia coli* mutant, *Meinhart and Simmonds*, 329
Steroid(s): Cortico-. *See* Corticosteroids
Hydroxy-. *See* Hydroxysteroid
Metabolism, *Burstein and Dorfman*, 581
Wotiz, Richterich-van Baerle, and Lemon, 969
16-Substituted, *Huffman and Lott*, 343
17-Trioxy-. *See* Trioxysteroid
Urine, glucuronidase hydrolysis, *Engel, Carter, and Fielding*, 99
Streptococcus: Fatty acids, *Hofmann and Tausig*, 415
Streptokinase: Plasminogen, effect, *Troll and Sherry*, 881
Succinic acid: Ureido-. *See* Ureidosuccinic acid
Sucrose: Monosaccharide conversion to, wheat seedlings, *Edelman, Ginsburg, and Hassid*, 843
Synthetase: Glutathione. *See* Glutathione synthetase

T

Taurine: Sulfur 35-labeled, metabolism, diet effect, *Portman and Mann*, 733
Taurocholate: Sulfur 35-labeled, metabolism, diet effect, *Portman and Mann*, 733
Testosterone: Metabolism, blood serum effect, *Wotiz, Richterich-van Baerle, and Lemon*, 969
Thiamine: Analogues, *Cerecedo and Eich*, 893
Neopyri-. *See* Neopyrithiamine
Oxyneopyri-. *See* Oxyneopyrithiamine
Thioctic acid: Removal, enzymes, *Seaman and Naschke*, 705
Thyroid: Cholesterol, radioactive, fate, effect, *Weiss and Marx*, 349
Iodine, inorganic, labeled, conversion to iodine, organic, labeled, effect, *Taurog, Potter, and Chaikoff*, 119
Thyrotropic hormone: Pituitary, anterior, purification, *Fels, Simpson, and Evans*, 311
Thyroxine: Homologues, iodine exchange, *Gleason*, 837
Torulopsis utilis: Amino acid biosynthesis, *Abelson and Vogel*, 355
Trioxysteroid(s): 17-, urine, determination, *Rivoire, Rivoire, and Poujol*, 11
Trypsin: Chymo-. *See* Chymotrypsin
Inhibitors, chymotrypsin, action, *Wu and Laskowski*, 609
Tryptophan: Acetate, radioactive, conversion to, *Aerobacter aerogenes, Rafelson*, 479
Acetyl-D-. *See* Acetyl-D-tryptophan
Acetyl-L-. *See* Acetyl-L-tryptophan
Glucose, radioactive, conversion to, *Aerobacter aerogenes, Rafelson*, 479
Tumor: -Bearing mice, nucleic acid pyrimidines, ureidosuccinic acid as precursor, *Anderson, Yen, Mandel, and Smith*, 625
See also Carcinoma, Neoplasm

Tyrosine: *o*-, metabolism, phenylketonuria, *Armstrong and Shaw*, 805
Requirements, *Armstrong*, 409

U

Ureidosuccinic acid: Nucleic acid pyrimidines, precursor, tumor-bearing mice, *Anderson, Yen, Mandel, and Smith*, 625
Uric acid: Oxidation, oxonic acid and allantoxaidin relation, *Canellakis and Cohen*, 379
—, peroxidases, effect, *Canellakis, Tuttle, and Cohen*, 397
—, uricase effect, *Canellakis and Cohen*, 385
Uricase: Uric acid oxidation, effect, *Canellakis and Cohen*, 385
Uridine nucleotide: 5′-, phosphorylation, liver, *Herbert, Potter, and Takagi*, 923
Urine: Corticosteroids, isolation and determination, scurvy, *Burstein, Dorfman, and Nadel*, 597
Corticosterone metabolites, *Engel, Carter, and Fielding*, 99
Proteolytic inhibitor, anticoagulant properties, *Shulman*, 655
Steroids, glucuronidase hydrolysis, *Engel, Carter, and Fielding*, 99
Urine—*continued:*
17-Trioxysteroids, determination, *Rivoire, Rivoire, and Poujol*, 11

V

Vitamin: B_6 analogues, enzyme action, *Olivard and Snell*, 203, 215
— —, growth effect, *Olivard and Snell*, 203, 215
B_{12}, choline methyl group biosynthesis from methanol, effect, *Verly and Cathey*, 621

W

Wheat: Germ, carbon dioxide fixation into oxalacetate, enzymatic, *Tchen and Vennesland*, 533
Seedlings, monosaccharide conversion to sucrose and cellulose, *Edelman, Ginsburg, and Hassid*, 843

Y

Yeast: Glutathione synthetase, isolation and properties, *Snoke*, 813
Pyruvic acid, carbon 14-labeled, conversion to monoamino acids, *Wang, Christensen, and Cheldelin*, 365
See also Torulopsis

7543

on,
11

ion,
215
ell,
215
esis
and
621

tion
chen
533
sion
nan,
843

tion
813
con-
ang,
365